AN APPROACH FOR THE PREVENTION OF THALASSAEMIA IN PAKISTAN

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ABSTRACT

The basic aim of this thesis was to identify a suitable approach for prevention of thalassaemia in Pakistan. The overall carrier rate for β-thalassaemia was 5.3%. The rate varied by ethnic group, i.e. Punjabi: 4.5%, Pathan: 5.2%, Sindhi: 1.3%, Baluchi: 9% and Mohajir: 5.2%. The annual birth rate of affected children was estimated at 1.35 per 1000. Screening for α-thalassaemia showed 6% carrier rate for $\alpha^{-3.7}\alpha/\alpha\alpha$ genotype. Approaches for identifying carriers and at risk couples were investigated in pregnant females, but proved to be technically difficult and cost ineffective. By contrast screening in ten index families with haemoglobin disorders identified 21-70% (mean 31%) carriers per family. No carrier was identified in five control families without a history of haemoglobin disorder. Follow-up for one year in the families screened showed a significant effect on the marriage choices.

Molecular basis of thalassaemia was investigated in 1240 mutant alleles in all ethnic groups. 19 different mutations were found including one novel allele. The five commonest mutations accounted for 81% of the alleles. The pattern of mutations was significantly different in the ethnic groups studied. 7.5% of patients on blood transfusions had thalassaemia intermedia, and its genetic basis included Xmn-1 +/+ genotype (36%) and mild mutations (31%). Coincidental α-thalassaemia was found or suspected in 38% of cases of thalassaemia intermedia. Prenatal diagnosis of thalassaemia was introduced for the first time in Pakistan and in the two years of the study 158 couples used the test. All couples, except two, already had at least one affected child. They had better education and socio-economic status than the controls and 93% requested termination of pregnancy when the fetus was affected. Over 98% of the diagnoses were done by direct mutation analysis. A multiplex polymerase chain reaction for mutation analysis was developed and significantly reduced the total cost and time required for prenatal diagnosis.

Consanguineous marriage and recessive disorders were studied in the nine index and the five control families. In 319 couples studied 46% were consanguineous, 52% were Biradri/Tribe members, and only 6% were completely unrelated. Consanguineous marriage (2^{nd} cousins or closer) had increased from 12% in the 1st generation to 45% in

the 2nd generation. Antecedent consanguinity had significant effect on the kinship coefficient of the close as well as distant relatives. There was only a marginal difference in coefficient of inbreeding, calculated in a random population sample by conventional methods (0.0257) and by allele frequencies at the D21S11 locus (0.0272). Morbidity and mortality from genetic causes was significantly higher in the children of consanguineous than the non-consanguineous couples living under similar socio-economic conditions.

This pilot study appears to have identified a suitable approach for prevention of thalassaemia in Pakistan.

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ABREVIATIONS USED

A	Adenine
AFIP	Armed Forces Institute of Pathology
ARMS	Amplification Refractory Mutation System
bp	base pairs
C	Cytosine
Cd	Codon
CI	Confidence Interval
CVS	Chorionic Villus Sampling
Del	Deletion
DGGE	Denaturing Gradient Gel Electrophoresis
dl	decilitre
DNA	Deoxy-ribo Nucleic Acid
dNTP	deoxynucleoside triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EMR	Eastern Mediterranean Region
fl	femtolitre
Fr	Frame shift
G	Guanine
g	gram
Hb	Haemoglobin
HIV	Human Immune Virus
ID	Identification
IVS	Intervening Sequence
LCR	Locus Control Region
MCH	Mean Cell Haemoglobin
MCV	Mean Cell Volume
mRNA	messenger Ribo Nucleic Acid
µl	microlitre
ng	nanogram
NWFP	North West Frontier Province

PCR	Polymerase Chain Reaction
pg	picogram
pM	picomole
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo Nucleic Acid
Rs	Rupees
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SSCP	Single Strand Conformation Polymorphism
STR	Short Tandem Repeat
T	Thymidine
Taq	Thermus aquaticus
Thal	Thalassaemia
TI	Thalassaemia Intermedia
TM	Thalassaemia Major
TRBC	Total Red Blood Cell Count
UCH	University College Hospital
UV	Ultra Violet
WHO	World Health Organization
yrs	years

1 Introduction

Globin genes:

Haemoglobin contains two α-like globin chains and two β-like globin chains, each forming a complex with one molecule of haem. The globin chains are encoded by α and β-globin gene clusters located on chromosomes 16 and 11 respectively (Thein and Weatherall 1988).

α-globin gene cluster:

Human α-globin cluster resides near the telomere of the short arm of chromosome 16 and encompasses approximately 30 kb (Fig: 1.1). It includes an embryonic gene (ζ), two adult genes (α1 and α2), three pseudogenes (ψζ1, ψα2, ψα1) and a gene of unknown significance (θ1) (Higgs et al, 1989).

β-globin gene Cluster:

The β-globin gene cluster spans approximately 60 kb on the short arm of chromosome 11 (Fig-1.1). It is composed of an embryonic gene (ε), a duplicated fetal gene (γ), a pseudo β-gene, a minor adult gene (δ) and the adult β-gene (Bunn and Forget 1986).

Structure of globin genes:

Throughout the vertebrates, the basic intron-exon structure of the globin genes remains constant. The globin genes are compact (1 to 2 kb) and have three coding regions called exons that are interrupted by two intervening sequences (IVS) of variable length called introns (Collins and Weisman 1984). The two IVS regions in the α-globin genes are located between codons 31 and 32 and between codons 99 and 100. Whereas, in the β-globin genes the IVS regions interrupt the sequences between codons 30 and 31 and between codons 104 and 105 (Fig: 1.1) (Thein and Weatherall 1988). The IVS-I is shorter than the IVS-II in both α and β-genes whereas the IVS-II in α-genes is considerably shorter than in the β-globin genes.

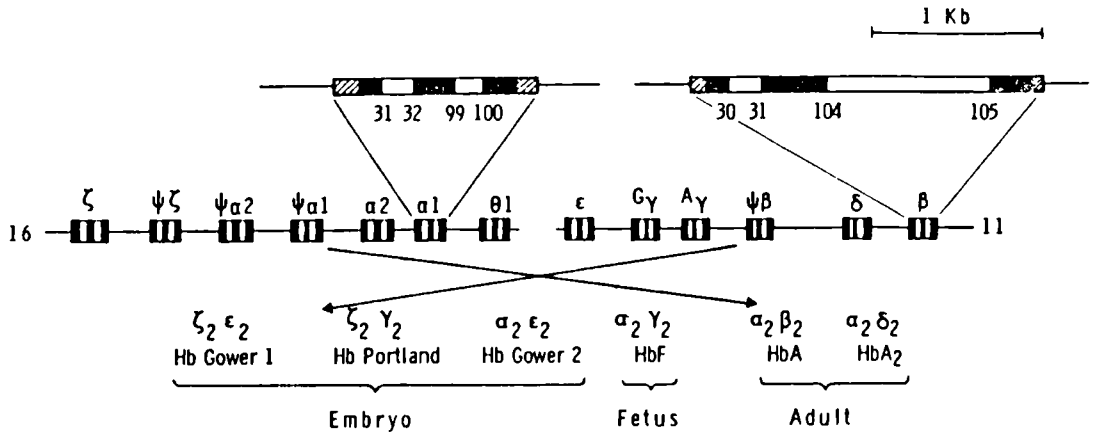


Fig: 1.1. Organization of the human α and β-globin gene clusters on chromosome 16 and 11. The genetic control of various embryonic, fetal and adult haemoglobins is also shown. Hatched boxes in the individual genes represent 3' and 5' untranslated regions, black boxes represent exons and the white boxes represent intervening sequences (IVS) (Thein and Weatherall 1988).

Globin gene expression:

The α- and β-globin loci are activated towards the end of the third week of gestation. There is a transition during development from the production of embryonic haemoglobin (Hb Portland, $\zeta_2\gamma_2$; Hb Gower-1 $\zeta_2\varepsilon_2$; Hb Gower-2 $\alpha_2\varepsilon_2$) to fetal haemoglobin (Hb-F, $\alpha_2\gamma_2$) to adult haemoglobins (Hb-A, $\alpha_2\beta_2$; Hb-A_2, $\alpha_2\delta_2$) (Fig: 1.1) (Peschle et al, 1985). The α-globin gene expression remains constant throughout life. The protein products of α1- and α2-genes are identical, however, the steady state level of α2- mRNA predominates over the α1- mRNA by approximately 3:1 (Liebhaber et al, 1986). On the other hand ζ,-, ε-, and γ-globin gene expression is down-regulated and β-globin gene expression increases with the transition from fetal to adult haematopoiesis. The γ to β-globin switching is normally complete by six months after birth (Cunningham and Jane1996).

Temporal control:

The globin genes are expressed in a developmental sequence. As development proceeds the site of erythropoiesis shifts from the yolk sac in the early embryo, to the liver in fetal life, and finally to the bone marrow in the postnatal life (Weatherall and Clegg 1981). Tuan et al, (1985) observed that *cis*-acting erythroid specific DNAase-I hypersensitive sites are present in a broad region of more than 20 kb 5' to the ε-globin gene. The DNAase-I hypersensitive region confers a high level, position independent, expression on linked globin genes (Grosveld et al, 1987). The term Locus Control Region (LCR), has been used to describe the cis-acting sequences responsible for this effect (Orkin 1990). It is postulated that temporal regulation of β-like globin genes results from competition between embryonic, fetal and adult globin genes for interaction with a common LCR (Townes and Behringer 1990).

Trans-acting factors like GATA binding proteins, synthesized in the yolk sac, fetal liver and bone marrow, may bind to a DNA sequence motif (T/A)GATA(A/G) present in the ε, γ and β-globin promoters for the ordered expression of the respective genes (Orkin 1990). Two such proteins, GATA-1 and GATA-2 have been shown to be essential for transcriptional control of erythroid specific gene expression (Shivdasani and Orkin 1996).

Existence of an LCR for the α-globin cluster has been suggested in the sequences upstream from the ζ-globin gene (Higgs et al, 1990a).

Promoter function:

The region located upstream to the coding sequence of many structural genes which controls individual gene expression and provides a site for binding of RNA polymerase is called promoter region. At least three preserved definable elements contribute in varying degrees to the promoter function of globin genes (Collins and Weisman 1984). The first preserved 5' untranslated sequence is the "TATA" or "ATA" box located approximately 25-30 bp upstream from the initiation site in both α-like and β-like globin genes (Fig 1.2). The "ATA" box is thought to play an important role in locating the site at which the transcription initiates. The second important component is the "CCAAT" box located approximately 80 bp upstream

from the transcription initiation site. Its position is somewhat more variable than the "ATA" box. The third component of the promoters is the "GGGGYG" (Y: a pyrimidine nucleoside) or the inverted type "CRCCC" (R: a purine nucleoside) sequence that is located 90 bp upstream from the initiation site (Collins and Weisman 1984). The normal human δ-globin gene does not have a perfectly conserved "CCAAT" sequence and this, in part, may be responsible for the low normal expression of the gene (Bunn and Forget 1986).

Transcription:

The transcription initiation site for globin genes correspond to the Cap site located 50 bp upstream from the initiation codon (AUG) which also marks the 5' end of the mature cytoplasmic mRNA (Fig: 1.2). The transcription goes on to include the exons introns and to the sequences beyond the highly conserved 3' "AATAAA" polyadenylation site (Collins and Weisman 1984).

Post transcriptional processing:

Following transcription the 5' end of the transcript is capped by addition of a methylated guanylic acid residue. This process is essential to prevent exoneucleolytic degradation of the nascent transcript (Nevins, 1983). The mRNA transcript is also cleaved and subsequently polyadenylated at a site marked by the "AAUAAA" sequence (Collins and Weisman 1984). Another important posttranscriptional event is splicing in which the introns are removed from the transcript. The process begins with cleavage of the 5' junction between exon and the intron. This results in looping back of the 5' end of the intron to form a 5'-2' phosphodiester bond with an "A" residue about 30 nucleotides 5' to the 3' splice junction (Padgett et al, 1984). The resulting structure is called a lariat. The first two nucleotides at both 5' and 3' end of the intron are essential for splicing. Nearly all eukaryotic genes have a highly conserved "GT" sequence at the 5' end and "AG" sequence at the 3' end of every intron. Marked similarities in sequence have been observed at the exon-intron boundaries in all eukaryotic genes. These identified sequences are called consensus sequences. For donor splice sites (5' consensus sequence) they include the last 3 nucleotides of the exon and the first 6 nucleotides of the intron and for the acceptor splice sites (3' consensus sequence) they are the last 10 nucleotides of the intron and the first nucleotide of exon (Shapiro and Senapathy, 1987).

CAATTTCTTATTTGTGTAATAAGAAAATTGGGAAAACGATCTTCAATATGCTTACCAAGCTGTGATTCCAAATATTACGTAAATACACTTGCAAAG
GAGGATGTTTTTAGTAGCAATTTGTACTGATGGTATGGGGCCAAGAGATATATCTTAGAGGGAGGGCTGAGGGTTTGAAGTCCAACTCCTAAGCCA
GTGCCAGAAGAGCCAAGGACAGGTACGGCTGTCATCACTTAGACCTCACCCTGTGGAGCCACACCCTAGGGTTGGCCAATCTACTCCCAGGAGCAG
GGAGGGCAGGAGCCAGGGCTGGGCATATAAGTCAGGGCAGAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAA
ACAGACACCATG**GTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTG**
GGCAG*GTTGGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGAAACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTTCTGATAGGCAC*
*TGACTCTCTCTGCCTATTGGTCTATTTTCCCACCCTTAG***GCTGCTGGTGGTCTACCCTTGGACCGAGAGGTTCTTTGAGTCCTTTGGGGATCTGTC**
CACTCCTGATGCTGTTATGGGCAACCCTAAGGTGAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCT
CAAGGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGATCCTGAGAACTTCAGG*GTGAGTCTATGGGACCCTTGATGTTT*
TCTTTCCCCTTCTTTTCTATGGTTAAGTTCATGTCATAGGAAGGGGAGAAGTAACAGGGTACAGTTTAGAATGGGAAACAGACGAATGATTGCATC
AGTGTGGAAGTCTCAGGATCGTTTTAGTTTCTTTTATTTGCTGTTCATAACAATTGTTTTCTTTTGTTTAATTCTTGCTTTCTTTTTTTTTCTTCT
CCGCAATTTTTACTATTATACTTAATGCCTTAACATTGTGTATAACAAAAGGAAATATCTCTGAGATACATTAAGTAACTTAAAAAAAAACTTTAC
ACAGTCTGCCTAGTACATTACTATTTGGAATATATGTGTGCTTATTTGCATATTCATAATCTCCCTACTTTATTTTCTTTTATTTTTAATTGATAC
ATAATCATTATACATATTTATGGGTTAAAGTGTAATGTTTTAATATGTGTACACATATTGACCAAATCAGGGTAATTTTGCATTTGTAATTTTAAA
AAATGCTTTCTTCTTTTAATATACTTTTTTGTTTATCTTATTTCTAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCA
TGCCTCTTTGCACCATTCTAAAGAATAACAGTGATAATTTCTGGGTTAAGGCAATAGCAATATTTCTGCATATAAATATTTCTGCATATAAATTGT
AACTGATGTAAGAGGTTTCATATTGCTAATAGCAGCTACAATCCAGCTACCATTCTGCTTTTATTTTATGGTTGGGATAAGGCTGGATTATTCTGA
*GTCCAAGCTAGGCCCTTTTGCTAATCATGTTCATACCTCTTATCTTCCTCCCACAG***CTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACT**
TTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCTAATGCCCTGGCCCACAAGTATCACTAAGCTCGCT
TTCTTGCTGTCCAATTTCTATTAAAGCTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCT
GCCTAATAAAAAACATTTATTTTCATTGCAATGATGTATTTAAATTATTTCTGAATATTTTACTAAAAAGGGAATGTGGGAGGTCAGTGCATTTAA

Fig: 1.2. Sequence of the β-globin gene. The sequences important for promoter function, capping, initiation, termination and polyadenylation are underlined. The exons are represented as bold and the introns are shown as italics (After Collins and Weisman 1984).

Translation:

Once processed the mRNA transcript is transported out of the nucleus and acts as a template on which the amino acid residues are sequentially added to form the polypeptide. The process is initiated at the initiation codon (AUG) and is terminated when a stop codon (UAA) is encountered (Bunn and Forget 1986).

Thalassaemia:

Historical background:

(Bunn and Forget 1986):

Thomas Cooley gave the first description of thalassaemia in 1925. He described a syndrome among children of Italian decent characterized by profound anaemia, splenomegaly, and bony abnormalities. The term "thalassaemia" derived from the Greek θαλασσα (the sea) was introduced by Whipple and Bradford in 1932 to show the apparent association between the disease and the Mediterranean. A number of Italian workers, between 1925 and 1935 described the syndrome of mild anaemia and increased osmotic resistance of red cells, who described it as "la malattia di Rietti-Greppi-Micheli". In 1936 Caminopetros in Greece and in 1937 Angelini in Italy concluded that the Cooley's anaemia is a recessively inherited disorder resulting from homozygous inheritance of the Rietti-Greppi-Micheli syndrome.

Definition and classification:

Thalassaemias are a heterogenous group of genetic disorders of haemoglobin synthesis, that result from a reduced rate of production of one or more of the globin chain(s) of haemoglobin (Weatherall 1996). They are divided into the α-, β-, δβ-, or γδβ-thalassaemias according to the type of chain(s) affected (Table: 1.1). The quantitative reduction in globin chain synthesis in thalassaemia syndromes differentiates these from the structural changes seen in abnormal haemoglobins. The structurally abnormal haemoglobins are mostly produced in normal amounts. However, some abnormal haemoglobins, Hb-E and Hb-Knossos for example, are also associated with reduced globin chain production.

Table: 1.1 Classification of thalassaemias (Weatherall et al, 1989; Weatherall 1996).

α-Thalassaemias:
- α^{o}-Thalassaemias
- α^{+}-Thalassaemias
  - Deletion
  - Non-deletion
- With α-chain Hb variants
- With β-chain Hb variants
- With β-Thalassaemia

β-Thalassaemias:
- β^{o}-Thalassaemias
- β^{+}-Thalassaemias
- With β-chain Hb variants
- With α-chain Hb variants
- With α-Thalassaemia

δβ-Thalassaemia:
- $(\delta\beta)^{o}$-Thalassaemia
- $(^{A}\gamma\delta\beta)^{o}$-Thalassaemia
- $(\varepsilon\gamma\delta\beta)^{o}$-Thalassaemia
- δ-Thalassaemia
- γ-Thalassaemia

Hereditary Persistence of Fetal Haemoglobin (HPFH):
- Deletion
  - $(\delta\beta)^{o}$-HPFH
- Non-deletion
  - Linked to β-globin gene cluster
  - Unlinked to β-globin gene cluster

World wide distribution of thalassaemia:

Disorders of haemoglobin are considered to be the most common genetic disorder world wide. About 250 million people (4% of the world population) carry a gene for an abnormal haemoglobin (WHO 1993). α^{+}-thalassaemia is the commonest globin mutation. Each year about 240,000 infants are born with a major haemoglobin disorder. Sickle cell disease accounts for nearly 78% of the affected births, β-thalassaemia major or Hb-E/β-thalassaemia accounts for about 20%, and about 1.6% is accounted for by Hb-S/β-thalassaemia (WHO 1985).

The disease has a high prevalence in a broad belt including the Mediterranean the Middle East, Indian subcontinent and the South East Asia (Weatherall and Cleg 1981). The highest concentration of α-thalassaemia is found in the Southeast Asia where up to 17,000 severely affected cases are born every year (WHO 1987).

Population genetics of thalassaemia:

The world distribution of thalassaemia and other major abnormal haemoglobins coincides with that of Plasmodium falciparum malaria. It is thought that heterozygotes are resistant to falciparum malaria and the selective advantage this provides compensates for the continual loss through the death of the homozygotes (Weatherall and Clegg 1981). The high frequency of thalassaemia genes, in populations where these are prevalent, exist in a state of balanced polymorphism due to heterozygote advantage on one hand and continuing loss through homozygotes on the other hand (Modell and Berdoukas 1984).

The heterozygote advantage against falciparum malaria may be the result of an increased susceptibility to red cell damage by oxidative stress in thalassaemia that leads to premature destruction of the parasite (Friedman et al, 1981). A strong correlation between the degree of parasite invasion and the lack of deformability of thalassaemic erythrocytes may also contribute to resistance against invasion by malarial parasite (Weatherall et al, 1989).

Molecular pathology of α-thalassaemia:

Most α-thalassaemias are caused by large gene deletions. Two α-thalassaemia phenotypes are recognized (1) α°-thalassaemia or α-thalassaemia-1 is characterized by complete absence of α-globin chains and (2) α^{+}-thalassaemia or α-thalassaemia-2 in which there is mild reduction in α-globin synthesis. α°-thalassaemia results from deletions that involve two α-globin genes (--/αα), whereas α^{+}-thalassaemia usually results from deletion of one α-globin gene (- α/αα) (Higgs et al, 1990b).

α-thalassaemia-2 is the commonest type of thalassaemia. It has a frequency of 30% in some parts of Africa (Dozy et al, 1979) and 30-50% in some parts of India (Kulozik et al, 1988; Gupta et al, 1991). The commonest α-thalassaemia-2 determinant ($-\alpha^{3.7}$) results from deletion

of 3.7kb of DNA extending from IVS-II of α2-gene to the corresponding part of α1-gene. Another α-thalassaemia-2 determinant ($-\alpha^{4.2}$) results from deletion of 4.2kb of DNA extending from the 3' end of ψα-gene to the 3' end of α2-gene (Embury et al, 1980). Less common deletions causing α-thalassaemia-2 include $-\alpha^{3.5}$ (Kulozik et al, 1988).

α1 and α2-genes are embedded within two highly homologous 4 kb duplicated segments. These regions are divided into homologous sub-segments (X, Y, and Z) by non-homologous elements (I, II, and III). Reciprocal recombination (Fig: 1.3) between Z segments, which are 3.7kb apart, produces chromosomes with only one α-gene ($-\alpha^{3.7}$, rightward deletion) that causes α-thalassaemia (Embury et al, 1980) and others with three α-genes ($\alpha\alpha\alpha^{anti\ 3.7}$) (Goossens et al, 1980). The events can be subdivided, depending on exactly where within the Z box the crossover takes place, into $-\alpha^{3.7I}$, $-\alpha^{3.7II}$, and $-\alpha^{3.7III}$ (Higgs et al, 1984). Recombination between homologous X boxes, which are 4.2kb apart, gives rise to $-\alpha^{4.2}$-thalassaemia (Embury et al, 1980) and $\alpha\alpha\alpha^{anti4.2}$ chromosome (Lie-Injo et al, 1981).

α°-thalassaemia is caused by deletions of both α-globin genes (--/αα). A large number of such deletions have been reported in people from Southeast Asia and Mediterranean (Higgs et al, 1990b). These deletions appear to have resulted from illegitimate recombinations that are poorly understood (Lukens 1993).

Less commonly α-thalassaemia phenotype is caused by mutations affecting the function of intact gene. Non-deletional forms of α-thalassaemia have been reported in the Mediterranean, Southeast Asians, Indians, Blacks and Saudi Arabians (Higgs et al, 1990b; Baysal et al, 1995b). Most of the mutations occur in the dominant α2 gene that produces a more severe phenotype than mutations affecting the α1 gene (Higgs et al, 1989).

Molecular Pathology of β-thalassaemia:

In contrast to α-thalassaemia majority of β-thalassaemias are caused by point mutations affecting gene regulation or expression. The study of globin chain synthesis in thalassaemia homozygotes reveals two major types of β-thalassaemia (1) with some residual β-chain synthesis (β^{+}-thalassaemia) and (2) no β-chain synthesis (β°-thalassaemia) (Lukens 1993).

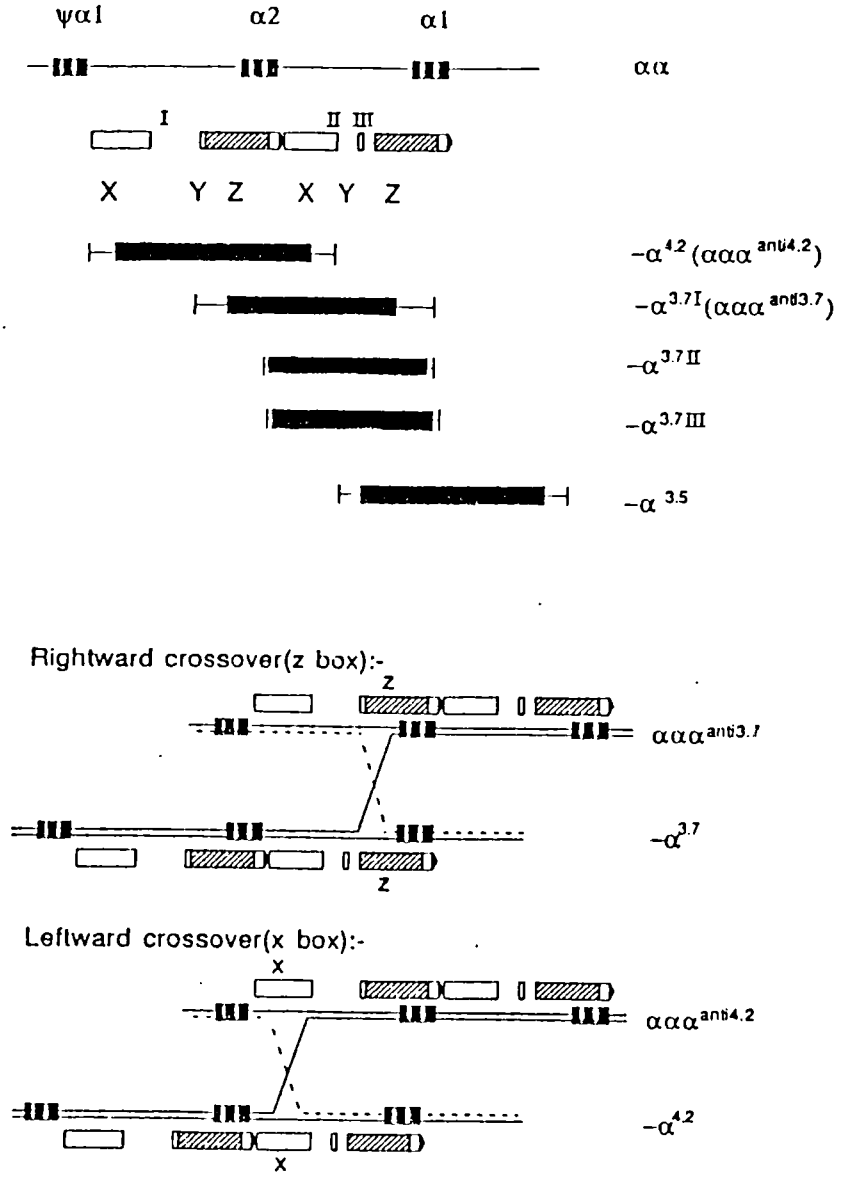


Fig: 1.3. The duplicated XYZ box arrangement containing the α genes. Nonhomologous regions (I, II, and III) are indicated. The extent of each deletion is indicated by the solid blocks and the limits of the breakpoints are represented by solid lines. Misaligned chromosomes crossing over to produce the $-\alpha^{3.7}$, $\alpha\alpha\alpha^{anti3.7}$ and $-\alpha^{4.2}$, $\alpha^{anti\ 4.2}$ haplotypes are also shown (Higgs et al, 1989).

Studies on the effect of thalassaemia mutations on globin gene expression demonstrate abnormal RNA processing (Triesman et al, 1983). Over 150 different mutations have been identified as a cause of β-thalassaemia (Baysal and Carver 1995). Studies on the

molecular genetics of thalassaemia in various ethnic groups have shown that each group tends to have its own set of common mutations (Kazazian et al, 1990). These mutations affect the gene expression by a variety of mechanisms.

Transcriptional promoter mutations:

The mutations resulting in reduced promoter function are mostly concentrated in the "TATA" box (Fig: 1.2) and in the proximal and distal "CACACCC" sequences at -90 and -105 neucleotides upstream of the gene (Kazazian et al, 1990). The promoter mutations tend to reduce the binding of RNA polymerase and lower the level of transcription to 20-30% of normal (Leukens 1993). The mutations in the promotor region of β-globin gene, for example C-T substitution at position -88 relative to Cap site (Orkin et al, 1984) produces a mild phenotype. A mutation at the Cap site of the β-globin gene also affects the transcription and capping with secondary effect on translation (Myers et al, 1986). Several mutations in the promoter region of β-globin gene are known to cause β^{+}-thalassaemia (Baysal and Carver 1995).

Chain termination mutations:

Premature termination of transcription may be caused by a nonsense mutation. A single nucleotide substitution in a codon, normally coding for an amino acid, can change it to a stop codon. CAG-TAG substitution at codon 39 is a classical example of nonsense codon (Trecartin et al, 1981). Premature chain termination may also be induced when the reading frame is shifted either by an insertion or deletion of nucleotide(s). The four nucleotide deletion (-TTCT) in codons 41 and 42, for example, results in premature introduction of a stop codon in the new codon 59 (Kimura et al, 1983). All chain termination mutants tend to produce β^{o}-thalassaemia phenotype (Lukens 1993). A large number of nonsense and frame-shift mutations have been documented as a cause of β^{o}-thalassaemia (Baysal and Carver 1995).

Mutations affecting RNA splicing:

The normal splicing may be disturbed by mutations involving the critical sequences at the exon-intron splice boundaries (Kazazian et al, 1990). Consensus sequence changes have been observed in donor site of IVS-1. G-C substitution at position +5, for example, results in

production of normally and abnormally spliced RNA that causes severe β^+-thalassaemia phenotype (Triesman et al, 1983). T-C substitution at position +6 of the IVSI, however, results in a milder β^+-thalassaemia phenotype (Orkin et al, 1982a). The altered mature RNA resulting from these mutations is ineffective as a messenger for β-globin synthesis. Nucleotide substitutions can lead to appearance of a consensus splice site sequence within an intron or an exon, the new splice site competing with normal splice sequences. G-A substitution at IVSI-110 creates a new acceptor splice site that resembles the normal acceptor splice site. The new site is used preferentially by the normal donor site that results in a β^+-thalassaemia phenotype (Spritz et al, 1981). A cryptic splice site, that mimics the consensus sequence for a splice site but is never used, may become used due to a mutation. A substitution at Codon 26 (Hb-E), for example, leads to use of a cryptic site at a low level of efficiency (Orkin et al, 1982b). At least 38 mutations result in abnormal RNA splicing and cause thalassaemia ranging in severity from mild β^+ to β^o-thalassaemia (Baysal and Carver 1995).

RNA Cleavage and polyadenylation mutants:

Six mutations in the polyadenylation site, all causing β^+-thalassaemia, have been reported (Baysal and Carver 1995). Mutations at the poly-A site, AATAAA-AACAAA in American Blacks for example, can result in cleaving of only a small percentage of the RNA transcript (Orkin et al, 1985). It is presumed that deficient β-globin synthesis is secondary to abnormal elongated transcripts (Kazazian et al, 1990).

Deletions as a cause of β-thalassaemia:

Deletions causing β-thalassaemia are rare. However, 13 different deletions of varying lengths have been reported (Baysal and Carver 1995). The commonest deletion removes 619 bp of IVS-II, exon-3 and sequences 3' of the gene (Spritz et al, 1982). Some deletions involving a large portion of the 5' untranslated region of β-globin gene are associated with an unusually high Hb-A_2 (Motum et al, 1992). Craig et al, (1992) have reported a 10.3kb deletion involving the 5' untranslated region of β-globin gene in an Asian Indian which is associated with an unusually high Hb-A_2 level. These mutations probably remove competition of β-globin gene promoters for the common Locus Control Region.

Dominant β-thalassaemia mutations:

A dominant form of β-thalassaemia, in which heterozygotes also become symptomatic, has been identified. A total of 27 different mutations, mostly involving the 3rd exon, are associated with this uncommon phenotype (Baysal and Carver 1995). At the molecular level they result from different mechanisms including a highly unstable β-variant as a result of single base substitution (Thein et al, 1991) or deletion of intact codons (Park et al, 1991), truncated β-chain due to premature termination, and an elongated β-globin with an altered carboxy terminal end as a result of frame-shift mutation (Thein et al, 1990). The dominant thalassaemia mutations in a heterozygous state form hyper-unstable haemoglobin variant that precipitate in the erythroid cells and lead to a syndrome of thalassaemia intermedia (Thein 1992).

β-thalassaemia due to unknown mutations:

Sometimes cases of typical β-thalassaemia are seen without any detectable mutation in the β-globin gene or its immediate flanking regions (Semenza et al, 1984; Kazazian et al, 1990). It is thought that these result from a mutation in another gene located elsewhere in the genome, which is important in β-globin gene expression (Cao et al, 1994).

World-wide distribution of β-thalassaemia mutations:

The spectrum of β-thalassaemia varies widely in different world populations. Each population has its own set of common mutations that account for most of the mutations. However, a large number of rare alleles are also observed in each ethnic population (Kazazian et al, 1990). Allele frequencies vary from one country to another or even between different regions of the same country (Rosatelli et al, 1992a). Because so many alleles are found in each region, many individuals with β-thalassaemia major carry two different alleles (are compound heterozygotes). However, in populations where the number of alleles is very limited or consanguineous marriage is common the incidence of true homozygotes is increased (Rosatelli et al, 1992b; Varawalla et al, 1991a). The prevalence of common mutations in various world populations is presented in Table: 1.2.

Table: 1.2. β-thalassaemia carrier rate and mutations in various world populations.

Country: (n) (Reference)	Carrier rate: (WHO 1985)	Mutations:	%
Italy: (914) (Rosatelli et al, 1992a)	2-15%	Cd 39 (C-T) IVSI-110 (G-A) IVSI-1 (G-A) IVSI-6 Others	40% 23% 10% 10% 17%
Greece: (642) (Loukopoulos 1996)	8%	IVSI-110 (G-A) Cd 39 (C-T) IVSI-1 (G-A) IVSI-6 (T-C) Others	43% 21% 14% 9% 13%
Sardinia: (3000) (Rosatelli et al, 1992b)	13%	Cd 39 (C-T) Fr 6 (-A) Fr 76 (-C) Others	96% 2% 1% 1%
Spain: (58) (Amselem et al, 1988)	<1%	Cd 39 (C-T) IVSI-6 (T-C) IVSI-110 (G-A) Others	64% 15% 9% 12%
Cyprus, (Turks): (232) (Baysal et al, 1992)	16-18%	IVSI-110 (G-A) IVSI-6 (T-C) IVSI-1 (G-A) IVSI-745 (C-G) Others	74% 8% 7% 7% 4%
Cyprus, (Greeks): (705) (Baysal et al, 1992)	16-18%	IVSI-110 (G-A) IVSI-6 (T-C) IVSI-1 (G-A) IVSI-745 (C-G) Others	80% 6% 5% 5% 4%
Turkey: (429) (Basak et al, 1992)	2-4%	IVSI-110 (G-A) IVSI-6 (T-C) Fr 8 (-AA) IVSI-1 G-A) Others	42% 10 5% 5% 38%
Malta: (32) (Sceri et al, 1993)	1.2-3%	IVSI-6 (T-C) Others	78% 22%
Azerbaijan: (135) (Kuliev et al, 1994)	6%	Fr 8 (-AA) IVSII-1 (G-A) IVSI-110 (G-A) Others	33% 21% 13% 33%
Bulgaria: (128) (Huisman 1990a)	3%	IVSI-110 (G-A) Cd 39 (C-T) IVSI-6 (T-C) IVSII-754 (C-G) Others	24% 22% 10% 10% 34%
Israel: (122) (Rund et al, 1990)	1-20%	Fr 44 (-C) IVSI-110 (G-A) -28 (A-C) IVSII-1 (G-A) Others	20% 18% 15% 14% 33%
Egypt: (48) (Novelletto et al, 1990)	2.5%	IVSI-110 (G-A) IVSI-6 (T-C) IVSI-1 (G-A) Others	27% 19% 10% 44%

Table: 1.2. (Contd...) β-thalassaemia carrier rate and mutations in various world populations.

Country: (n) (Reference)	Carrier rate: (WHO 1985)	Mutations:	%
Algeria: (172) (Labie et al, 1990)	5-6%	Cd 39 (C-T) IVSI-110 (G-A) Fr 6 (-A) IVSI-1 (G-A) Others	26% 25% 18% 15% 16%
UAE: (50) Quaife et al, 1994)	3%	IVSI-5 (G-C) Fr 8-9 (+G) IVSI -25 bp Others	66% 8% 8% 18%
Iran: (50) (Taghi personal communication)	2-4%	IVSII-1 (G-A) IVSI-5 (G-C) IVSI-6 (T-C) Others	22% 13% 9% 56%
North West Pakistan: (167) (Varawalla et al, 1991a)	2-6.5%	Fr 8-9 (+G) IVSI-5 (G-C) Fr 41-42 (-TTCT) Cd 15 (G-A) Others	35% 44% 10% 7% 4%
India: (535) (Varawalla et al, 1991a)	1-17%	IVSI-5 (G-C) Del 619 bp IVSI-1 (G-T) Fr 41-42 (-TTCT) Fr 8-9 (+G) Others	39% 25% 13% 10% 8% 5%
Burma: (170) (Brown et al, 1992)	β-Thal: 1-3% Hb-E: 1-25%	Hb-E IVSI-1 (G-T) IVSI-5 (G-C) Fr 41-42 (-TTCT) Others	41% 20% 16% 12% 11%
Thailand: (128) (Winichagoon et al, 1990)	β-Thal: 3-9% Hb-E: 0-40%	Fr 41-42 (-TTCT) Hb-E Cd 17 (A-T) IVSII-654 (C-T) Others	25% 19% 13% 6% 37%
China: (93) (Chan et al, 1987)	2-4%	Fr 41-42 (-TTCT) IVSII-654 (C-T) Cd 17 (A-T) -28 (A-G) Others	48% 22% 10% 8% 12%
Malaysia: (?) (Yang et al, 1989)	β-Thal: 1-10% Hb-E: 0-30%	IVSI-5 (G-C) Cd 19 (A-G) Fr 41-42 (-TTCT) Others	49% 15% 12% 24%
Indonesia: (72) Lie-Injo et al, 1989)	β-Thal: 3% Hb-E: 1-8%	IVSI-5 (G-C) Hb-E IVSII-654 (C-T) Others	44% 18% 10% 28%
Blacks: (38) (Gonzalez-Redondo et al, 1988)	1-3%	-29 (A-G) -88 (C-T) Cd 24 (T-A) Others	55% 21% 8% 16%

Pathophysiology of thalassaemia:

(Weatherall and Clegg 1981)

Quantitative reduction of one or more globin polypeptide chains has two major consequences: decreased haemoglobin synthesis and an imbalance between α and non-α chain production. The former is a major determinant of red cell hypochromia, but is of little clinical significance. Unbalanced globin chain production is the major determinant of the clinical outcome. In the absence of a complementary globin chain the free normal chains aggregate and precipitate within the cytoplasm, damage red cell membranes, and lead to premature destruction of erythroid cells. β-chain tetramers seen in α-thalassaemias (Hb-H) are unstable and lack the physiologically useful properties of normal haemoglobin. They tend to oxidize easily and precipitate within erythroid precursors. However, these tetramers remain soluble for a while and therefore result in a clinical syndrome that is not very severe.

In β-thalassaemia deficiency of β-chains result in accumulation of unpaired α-chains. Because of their great instability free α-chains aggregate to form insoluble inclusions in erythroid precursors. The burden of inclusions is sufficient to cause large scale ineffective erythropoiesis. The spleen traps inclusion bearing red cells that enter the circulation, where many incur irreparable mechanical and metabolic injury. In response to the ineffective erythropoiesis and shortened red cell survival there is an enormous increase in marrow activity. Expansion of the medullary cavities by erythroid tissue occurs at the expense of cortical bone.

Clinical and Laboratory features of α-thalassaemia syndromes:

Carriers of single α-gene deletion (-α/αα) are clinically normal. About half have mild hypochromia and microcytosis. Heterozygotes of α-thalassaemia-1 (--/αα) or homozygotes of α-thalassaemia-2 (-α/-α) usually do not have anaemia. They have distinct microcytosis and hypochromia (Huisman 1996). Hb-H disease (--/-α), on the other hand, has clinical picture of chronic haemolytic anaemia of variable severity. The syndrome of hydrops fetalis results from deletion of all 4 α-genes (--/--). The affected fetus is usually delivered prematurely and is either still born or dies shortly after birth (Lukens 1993). The haematological picture of different types of α-thalassaemias is summarized in Table: 1.3. Carrier detection for α-thalassaemia is difficult by routine haematological methods. Gene mapping by restriction endonucleases or

detection by PCR are reliable for detection of α-thalassaemia carriers (Kulozik et al, 1988; Baysal and Huisman 1994).

Table: 1.3. Summary of haematological findings in individuals with α-thalassaemia (Adapted from Higgs et al, 1989).

Phenotype	No of functional α-genes	Hb-Barts at birth (%)	Hb-H %	MCV	MCH	α/β chain synthesis ratio
Normal	4	0	0	85-100	~30	~1.0
α^+-thalassaemia trait	3	0-2	0	75-85	~26	~0.9
α°-thalassaemia trait	2	2-8	0 (occasional)	65-75	~22	~0.7
Hb-H disease	1	10-40	1-40 (many)	60-70	~20	~0.4
Hydrops fetalis	0	~80	+	110-120	low	0.0

Clinical and Laboratory features of β-thalassaemia syndromes:

Heterozygous β-thalassaemia (thalassaemia minor) is mostly asymptomatic but causes prominent abnormalities of red cells. A typical case has haemoglobin in the normal or lower normal range, MCV is ≤75 fl, MCH ≤25 pg, and Hb-A_2 is ≥3.5% (Lukens 1993). The most severe form of homozygous β-thalassaemia is recognized as thalassaemia major. The infant is normal at birth but usually within six months of age progressive pallor develops and the patient fails to thrive. In the absence of transfusion therapy haemoglobin concentration slowly falls to 3-5 g/dl and the characteristic clinical picture emerges. The child has a small stature, relatively large head, and moderate to marked hepato-splenomegaly (Modell and Berdoukas 1984). Table: 1.4 summarizes the clinical and haematological features of β-thalassaemia syndromes.

Thalassaemia intermedia:

The term Thalassaemia Intermedia (TI) is used to describe patients with the clinical picture of thalassaemia, which, although not transfusion dependent, is associated with a more severe degree of anaemia than that found in heterozygous carriers for α- or β-thalassaemia (Weatherall 1996). The intermediate forms of thalassaemia show a markedly heterogenous clinical picture. At the molecular level several factors may be responsible for the mild phenotype of thalassaemia that may exist in each patient either independently or in

combinations. These are summarized in Table: 1.5.

Mild β-thalassaemia mutations, for example C-T substitution at -88 (Orkin et al, 1984), C-T substitution at -101 (Gonzalez-Rodondo et al, 1989), and A-C substitution at the Cap site (Wong et al, 1987), when co-inherited with another severe β-thalassaemia mutation are often associated with a mild (intermedia) phenotype. Many of the promoter region mutations in heterozygous state are associated with minimal or no increase in Hb-A_2 level.

Table: 1.4. Clinical and haematological features of β-thalassaemia syndromes (Modified from Lukens 1993).

	Thalassaemia Major	**Thalassaemia Intermedia**	**Thalassaemia Minor**
Severity	++++	++	+,±
Genetics	Homozygous/double heterozygous	Homozygous/double heterozygous	Heterozygous
Splenomegaly	++++	++,+++	+, 0
Jaundice	+	++	0
Bony changes	++++,++	+, 0	+, 0
Haemoglobin	<7 g/dl	7-10 g/dl	>10 g/dl
Hypochromia	++++	+++	++
Microcytosis	+++	++	+
Target cells	10-35%	++	+
Stippling	++	+	+
Reticulocytes	5-15%	3-10%	2-5%
Nucleated RBC	+++	+, 0	0
Hb-F	20->94%	30-100%	1-2%
Hb-A_2	1-8.7%	<1-10.0%	3.5-8.0%

Table: 1.5. Molecular basis of thalassaemia intermedia (Cao et al, 1994).

1. *Mild defects of β-globin chain production*
    a. Homozygosity for mild β-thalassaemia
    b. Compound heterozygosity for mild and severe β-thalassaemia

2. *Homozygosity or compound heterozygosity for severe β-thalassaemia associated with*
    a. α-thalassaemia
    b. Genetic factors enhansing γ-chain production
        Gγ promotor mutation (homozygous Xmn-I polymorphism)
        Heterocellular HPFH

3. *β-thalassaemia due to large promoter region deletions*
4. *Homozygosity for δβ-thalassaemia or its combination with β-thalassaemia*
5. *Double heterozygosity for β-thalassaemia and triplicated α-globin gene*
6. *Heterozygosity for hyperunstable Hb variants (Dominant thalassaemia)*

Co-inheritance of α-thalassaemia in the form of deletion of two genes (--/αα or -α/-α) may raise the MCV and MCH in a β-thalassaemia heterozygote to a normal range (Melis et al, 1983). The severity of homozygous β-thalassaemia is also reduced by co-inheritance of two gene deletion α-thalassaemia (Cao et al, 1994). Co-inheritance of single α-gene deletion (-α/αα), however, affects the phenotype of only β^+-thalassaemia homozygotes (Gringras et al, 1994). Co-inheritance of triplicated α-genes (αα/ααα), on the other hand, may increase the severity of β-thalassaemia (Galanello et al, 1983).

Some patients of typical β-thalassaemia major produce larger quantities of Hb-F that compensates the globin chain imbalance created by lack of β-chain production. Studies in Sickle cell disease (SS) and β-thalassaemia heterozygotes have shown that A-T polymorphism at position -158 relative to $^{G}\gamma$ Cap site (recognized by Xmn-I) is associated with 3-11 fold increase in production per $^{G}\gamma$-globin gene (Gilman and Huisman 1985). Patients of homozygous β-thalassaemia associated with homozygous Xmn-I polymorphism at position -158 usually have a thalassaemia intermedia phenotype (Thein et al, 1987; Gringras et al, 1994).

Management of thalassaemia:

The mainstay of thalassaemia management is regular blood transfusions, iron chelation therapy and provision of other support facilities. A hyper-transfusion regimen that maintains a mean haemoglobin of 12 g/dl is recommended. Hyper-transfusion allows normal growth and physical activity, reduces marrow hyperplasia and the associated bony abnormalities and prevents splenomegaly and hypersplenism. The main disadvantage of hyper-transfusion is the accumulation of excess iron in the body that damages many organs including heart, liver and endocrine glands (Cao et al, 1992). Iron that accumulate over a period of time due to repeated blood transfusions and increased absorption from the intestine needs to be removed by regular use of iron chelators. Desferrioxamine is the most extensively used iron chelator (Giardina and Grady 1995). Its main disadvantage, other than the high cost, is a continuous subcutaneous route of administration. An oral iron chelator L1 is also available but the associated risk of agranulocytosis has limited its use (Brittenham 1992).

Splenectomy is considered for patients who require more than 200 ml of packed red cells/kg/year (Piomelli 1995). Thalassaemia patients on regular treatment protocols also require hormone replacement therapy (Kattamis and Kattamis 1995). Allogenic bone marrow transplantation is the only curative treatment available at present. Success rate of over 92% in selected cases has been achieved at some centres (Lucarelli et al, 1995). Reactivation of fetal haemoglobin production by 5-azacytidine or hydroxyuria may have some benefit in patients of thalassaemia intermedia (Olivieri 1996). The prospects of gene therapy, for the present, appear to be remote (Beuzard 1996).

The intermediate form of thalassaemia requires careful surveillance. The patients should be watched carefully in early childhood, and if there are signs of growth retardation or increasing bone deformity, they should be put on regular transfusions (Cao et al 1992). Patients of thalassaemia intermedia, who are kept off transfusion, usually develop splenomegaly that becomes a source of pooling of blood. In such patients splenectomy can be beneficial.

Thalassaemic children who are not given any treatment usually die before two years of age. Those who get adequate treatment reach their adult life. The prognosis of children who are

inadequately treated is poor.

Prevention of thalassaemia:

Although thalassaemia patients can be managed to a great extent by regular blood transfusions and other supportive measures, the high cost of treatment creates severe health burden in a community where the disorder is common. The burden has gradually increased because of the rise in life expectancy following introduction of modern supportive measures (Angastiniotis et al, 1986). Nowadays, therefore, a fundamental aspect of the management of these disorders is prevention. Several thalassaemia prevention programmes in the Mediterranean have shown that the birth rate of new thalassaemics can be brought down to almost zero (Cao 1987). The cardinal features of a thalassaemia prevention programme include (1) public education, (2) population screening, (3) genetic counselling, and (4) prenatal diagnosis (Fig 1.4).

Prerequisites:

The planning and organization of a comprehensive thalassaemia prevention programme has certain prerequisites. Political will and commitment by the government who is also the public health planner is essential. A basic situation analysis at the National level is necessary to determine priorities. In the absence of reliable epidemiological data preliminary information on trends and hospital statistics may be useful. The funding resources may be made available from governmental as well as non-governmental sources (Alwan and Modell 1997).

Public education:

All programmes for prospective control of thalassaemia in the Mediterranean had an intensive awareness campaign and involvement of population. Public education was achieved by means of mass media, posters, informative booklets, and by special teaching on thalassaemia in schools. More personal approaches through lectures and discussions with the community leaders of the population were very useful. The health care workers and the paramedical staff played a major role in implementation of the preventive programmes (Cao 1987). In addition to the public awareness campaign it is also essential to create an awareness amongst the state authorities about the possibility of an ever increasing expenditure on new cases of thalassaemia if no control measures are taken (Loukopoulos 1996).

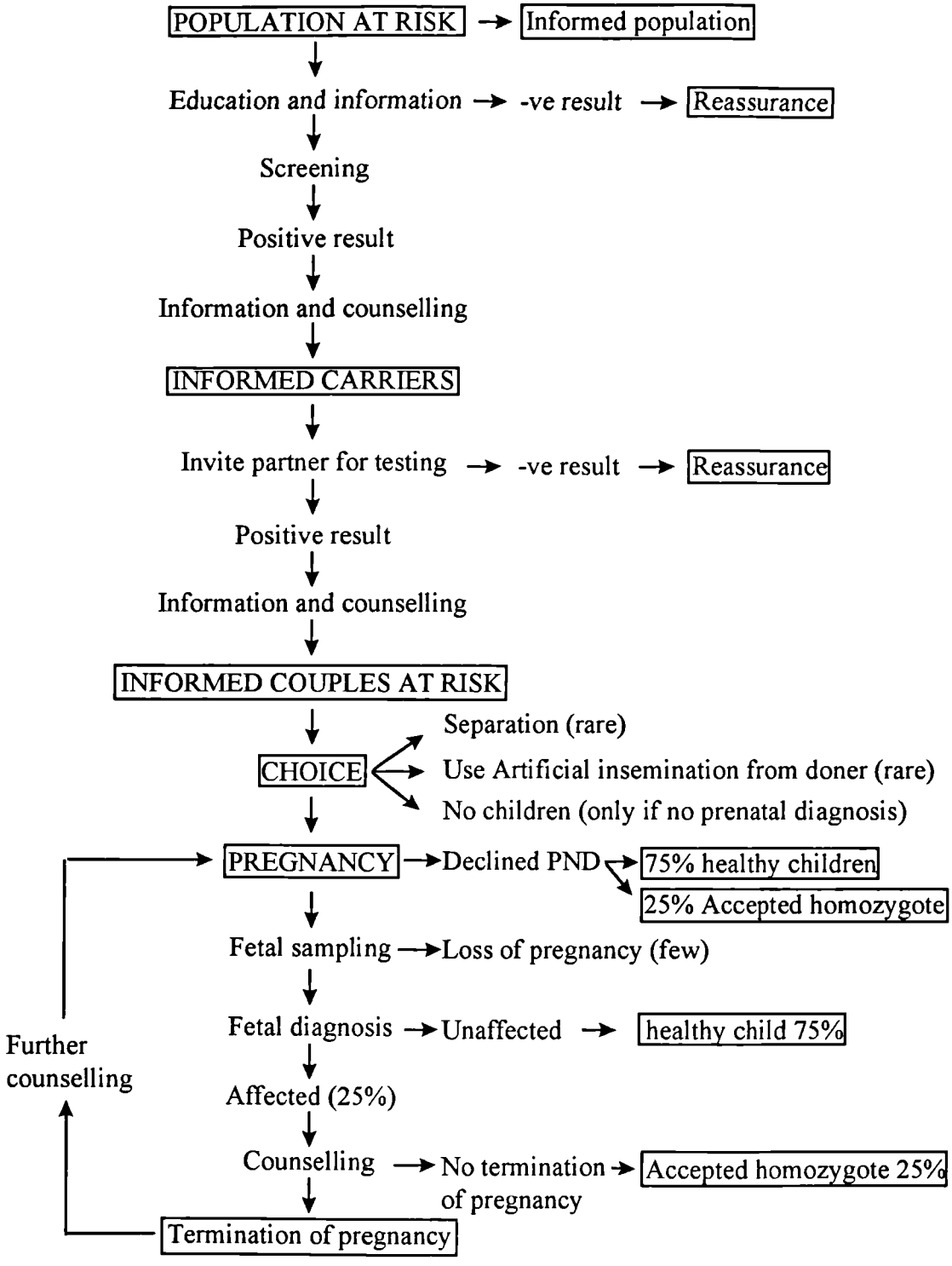


Fig: 1.4. An outline of the basic principles of thalassaemia prevention including public education, carrier screening, genetic counselling, and prenatal diagnosis (Based on Petrou et al, 1990).

Carrier screening:

Programmes designed to reduce the frequency of β-thalassaemia major births include the couples who already have an affected child or who are in their first pregnancy (WHO 1983). Retrospective diagnosis i.e. identification of a couple after the birth of an affected child, although very useful for individual couples, has only a limited effect on the number of affected children in the community (Cao 1987). The retrospective approach, however, may be useful in communities where final family size is large because a cessation of reproduction can reduce the birth rate of new thalassaemics to almost 50% (Alwan and Modell 1997). Initial programmes in the Mediterranean involved parents with an affected child and prospective diagnosis i.e. identification of the couples at risk before giving birth to an affected child, was instituted at a later stage (Cao et al, 1981). The choice of testing varies between premarital or at marriage as in Greece (Fessas 1986), mandatory screening of couples before marriage as in Cyprus (Angastiniotis et al, 1986) screening in the antenatle clinics as in the UK (Petrou 1994), or school leavers as in some areas of Italy (Bianco et al, 1985). An obvious disadvantage of screening during pregnancy is the finding of a couple at risk when the pregnancy is already advanced. Late testing may produce dramatic emotional stress on couples.

Screening the relatives of known carriers can also be an efficient approach for detecting thalassaemia. The approach can identify a large number of carriers (Mouzouras et al, 1980; Cao 1987). A useful and cost effective strategy adopted by several programmes is to test one member of a couple, the other member being tested when the first is found to be a carrier.

The carrier detection methods should be able to pick up β-thalassaemia trait, as well as δβ-thalassaemia, γδβ-thalassaemia, Hb-S, Hb-E, and Hb-C which, when co-inherited with β-thalassaemia, may result in thalassaemia syndromes. The methods should also be able to identify silent β-thalassaemia alleles and co-existing α- and β-thalassaemia trait (Cao et al 1994). A scheme (Fig 1.5), that can identify almost all carriers, should include red cell indices, haemoglobin electrophoresis, Hb-A_2 and Hb-F estimation, and globin chain synthesis studies (Modell and Berdoukas 1984). Molecular genetic studies can supplement the identification of silent β-thalassaemia carriers and those with co-existing α-thalassaemia (Cao et al, 1994).

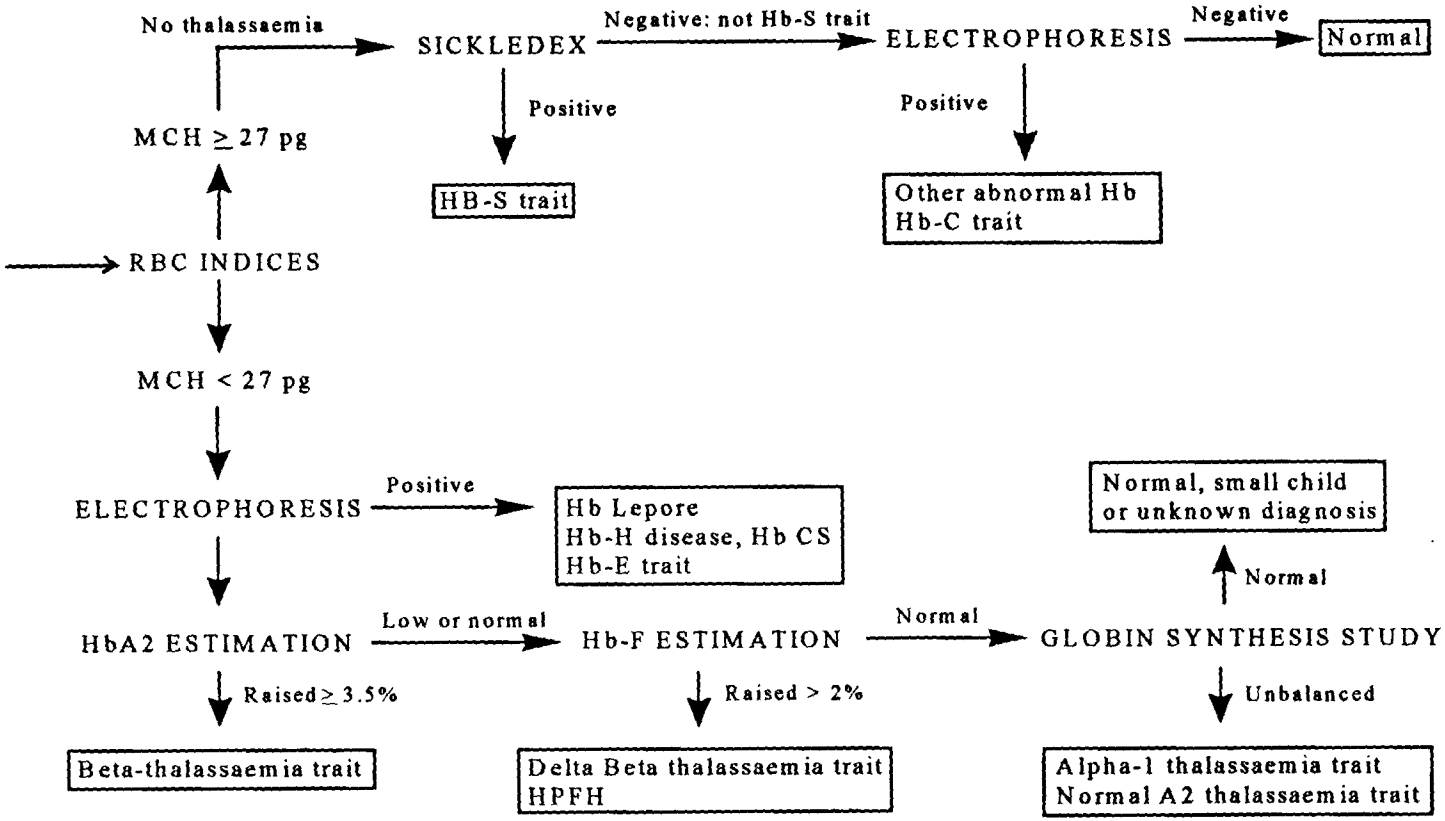


Fig: 1.5. Flow chart for identification of carriers of a haemoglobin disorder (Modell and Berdoukas 1984).

Data from various populations suggest that MCH is a more sensitive indicator than MCV for identifying unusual forms of β-thalassaemia (Modell and Berdoukas 1984). A cut off limit for MCH below which Hb-A_2 estimation should be done to confirm the diagnosis of thalassaemia trait may be 27 pg. An important diagnostic problem, especially in the Asians, arises from the high incidence of iron deficiency that is the commonest cause of severe microcytosis. Hb-A_2 level in β-thalassaemia trait with concomitant iron deficiency, however, remains in the higher range unless severe anaemia is present (Cao et al, 1994).

In large scale population screening for thalassaemia in a developing country red cell indices may be replaced by a cost effective one tube osmotic fragility test as a screening method and confirmation by Hb-A_2 estimation (Kattamis et al, 1981; Bianco and Silvestroni 1983).

Genetic counselling:

Genetic counselling aims at replacing misunderstandings about the cause of genetic disease with correct information and increasing people's control on their own and their family's health by informing them of the resources available for diagnosis, treatment and prevention. The basic principles of genetic counselling include autonomy of the individual or couple, right to complete information and highest standard of confidentiality (Harper 1993). The main components of genetic counselling are correct diagnosis in the presenting family member, explanation of the nature prognosis and treatment of the disorder, estimation of genetic risk for parents and family members, the options for avoiding genetic risks and supporting the individual or couple in making the right decision. The ethical principles governing genetic counselling need to be reviewed in the light of social and religious structures particularly in relation to the issues of prenatal diagnosis, and counselling in relation to customary consanguineous marriage (Alwan and Modell 1997).

Counselling is generally based on a private interview with the individual carrier or the couple. It should be non-directive and any mandatory measures restricting the individual freedom should be avoided because this may have negative effects (Angastiniotis and Hadjiminas 1986). In countries where high-risk ethnic minorities exist, in the UK for example, it may be difficult to provide adequate counselling because of problems of language, culture, and trust (Modell

and Berdoukas 1984). In a situation like this the services of a trained counsellor with the same cultural religious and linguistic background can be of great help (Petrou et al, 1990).

Prenatal diagnosis:

The basic objective of prenatal diagnosis is to give an informed choice to the at risk couple (Fig 1.4) (Petrou et al, 1990). The affected pregnancies are terminated only on the parent's request, the main consideration being for the future and quality of life of the child. The concept of prenatal diagnosis and termination of pregnancy has been accepted only slowly over the past 25 years and there is ongoing debate about its ethical and social implications. The decision whether or not to terminate an affected pregnancy is never taken lightly even in countries where prenatal diagnosis is fully accepted (Alwan and Modell 1997). Thalassaemia preventive programmes carried out before prenatal diagnosis was available and thus based solely on heterozygote detection and genetic counselling have not been effective in reducing the birth incidence of new cases of thalassaemia major (Barrai and Vullo 1980a). The introduction of prenatal diagnosis (Kan et al, 1975) gave a new dimension to thalassaemia prevention.

Prenatal diagnosis was initially done by globin chain synthesis ratio on fetal blood obtained in mid trimester gestation by fetoscopy or placental aspiration (Fairweather et al, 1978) and then by cordocentesis (Cao et al, 1986). With the development of sophisticated ultrasound scanners the practice has now shifted to first trimester fetal sampling by chorionic villus sampling (Ward et al, 1983). The rate of miscarriage with chorionic villus sampling is less than 1% (Centre for Disease Control and Prevention, Atlanta 1995). The laboratory diagnosis is now carried out by DNA analysis. Polymerase Chain Reaction (PCR) has simplified the process of mutation detection in the fetal tissue (Old et al, 1990).

In-vitro fertilization and embryo transfer has also enabled diagnosis to be carried out on just a few cells obtained from the embryo prior to implantation (Monk and Holding 1990). However, the present method of pre-implantation diagnosis are cumbersome, expensive and experimental, but if successful may be made acceptable reliable and simple (Alwan and Modell 1997).

Evaluation of thalassaemia control programmes:

The results of prevention programmes in various Mediterranean populations are summarized in Fig: 1.6. The best results have been achieved in Cyprus where the incidence has declined by almost 97%. This has been helped by a small size and population of the country, high standard of living, high literacy rate, low birth rate, high standard of health care and helpful attitude of the Church in endorsing premarital screening (Angastiniotis et al, 1986). In the UK, where the programme is active since 1977, the birth rate of thalassaemia major has fallen to just 50% of the expected. In the UK screening and counselling is well delivered to British Cypriots, but very inadequately to British Asians particularly Pakistanis (Modell et al, 1984). An important reason for the low acceptance rate for prenatal diagnosis amongst British Pakistanis and Bangladeshis is that they are referred late in the second trimester and many then refuse prenatal diagnosis. Due to the availability of first trimester diagnosis the acceptance rate of prenatal diagnosis has improved substantially in these communities (Petrou et al, 1990). Almost 80% of Pakistani couples referred in the first trimester now request prenatal diagnosis (Petrou 1994). Unfortunately, many such couples are identified late as the screening is done in antenatal clinics.

Cost effectiveness of thalassaemia prevention:

Cost analysis of prevention versus treatment, done in many Mediterranean countries and the UK, shows that the cost of prevention is equivalent to treating the affected new born for just one year (Ostrowsky et al, 1985; Cao 1987). Table: 1.6 shows six possible policies for thalassaemia (Alwan and Modell 1997). A comparison of the cost of the six policies in a population (Fig 1.7) shows that the policy involving best possible patient care, plus premarital and antenatal "prospective" carrier screening and genetic counselling, with availability of prenatal diagnosis is the most cost effective.

DNA analysis for thalassaemia:

In the mid 1970s, two revolutionary techniques became available that radically simplified the analysis of DNA structure. The first evolved from the discovery of DNA cleaving enzymes called restriction endonucleases which could cut a DNA molecule only at specific sequences (Weinberg 1985). The other technical revolution came with the ability to sequence DNA

(Sanger et al, 1977). The discovery of Polymerase Chain Reaction (PCR) in the mid 1980's has further simplified the analysis of DNA (Mullis and Faloona 1987).

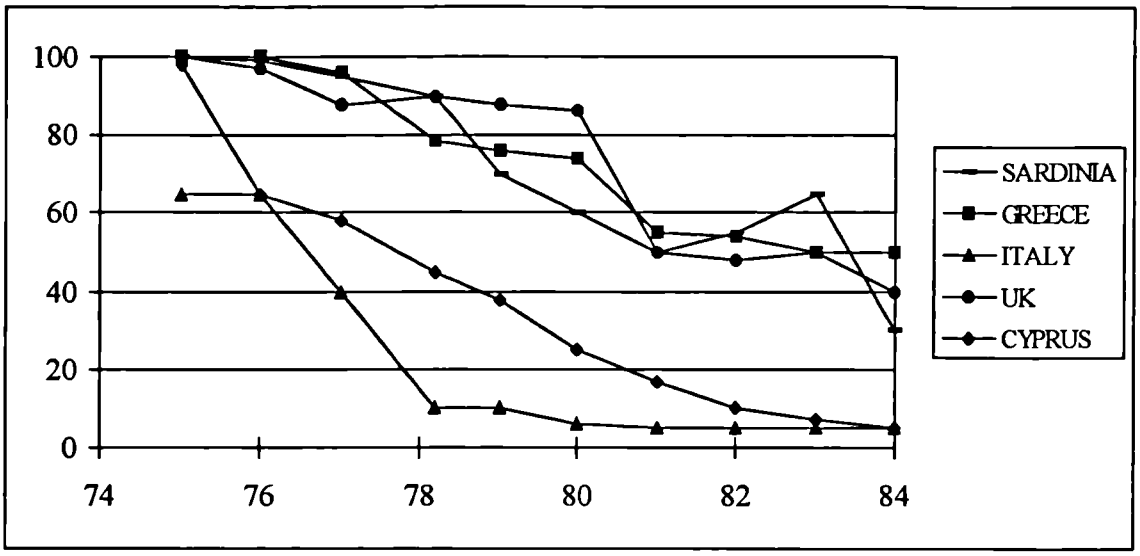


Fig: 1.6. Fall in the birth rate of infants with thalassaemia major associated with four national thalassaemia prevention programmes (Based on Cao 1987).

Table: 1.6. Six possible policies for thalassaemia. Policies 1-5 are currently in existence in different countries of the Eastern Mediterranean Region (EMR) (Alwan and Modell 1997).

Policy 1	A "baseline situation" in which no treatment, counselling or prevention is available. (Until recently this was the situation throughout most of the Middle East, but there has been rapid progression towards "policy 2" in many countries during the last 10 years.)
Policy 2	The best possible patient care, plus "retrospective" genetic counselling after the first affected child is diagnosed. (This is now common in many countries of the Region.)
Policy 3	The best possible patient care, plus "retrospective" genetic counselling, plus the option of prenatal diagnosis in subsequent pregnancies. (Available for a limited number of families in the Region at present.)
Policy 4	The best possible patient care including retrospective genetic counselling, plus "prospective" (premarital) carrier screening and counselling, but no prenatal diagnosis. This is in place in some countries of the EMR.
Policy 5	The best possible patient care, plus premarital and antenatal "prospective" carrier screening and genetic counselling, with availability of prenatal diagnosis. At present, the only country of the Region implementing this policy is Cyprus.
Policy 6	The best possible patient care, plus premarital, and family- and population-based "prospective" carrier screening, plus genetic counselling, but no prenatal diagnosis.

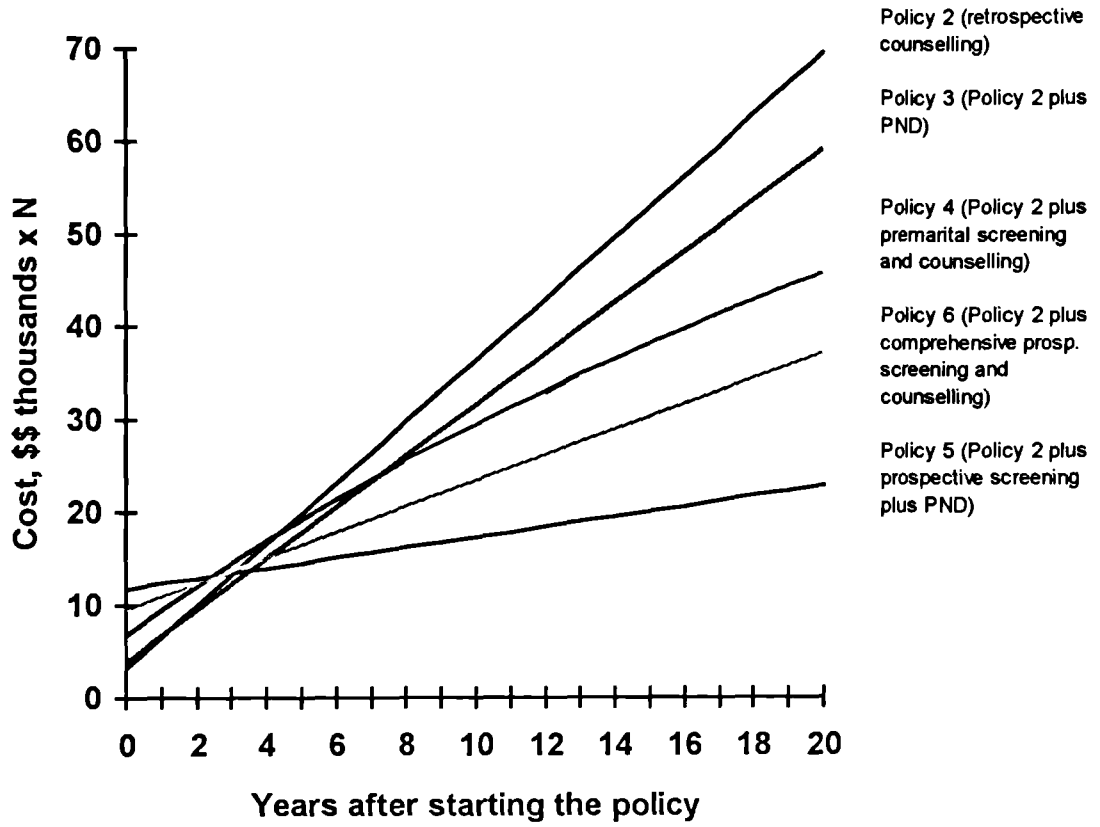


Fig: 1.7 Projected evolution of total costs of treating and preventing thalassaemia over the first 20 years, with the six different policies described in Table: 1.6 (Alwan and Modell 1997).

Southern blotting:

Localization of particular sequences within genomic DNA can be accomplished by the transfer technique of Southern (1975). Genomic DNA is digested with one or more restriction enzymes and the resulting fragments are separated according to size by electrophoresis through an agarose gel. DNA is then denatured in situ, transferred to nitrocellulose filter or nylon membrane and is then hybridized to radiolabelled probe. Southern blotting has been extensively used in the past for analysis of gene defects in thalassaemia (Boehm and Kazazian 1989).

Restriction Fragment Length Polymorphism (RFLP):

The term RFLP denotes a variation in fragment sizes when genomic DNA from different

individuals is cleaved with the same restriction enzyme. There are several polymorphic sites that are closely linked to the β-globin gene (Antonarakis et al, 1985). These polymorphic sites can be recognized by the use of restriction endonucleases (Fig: 1.8) which cleaves double stranded DNA at a specific sequence (Varawalla et al, 1992).

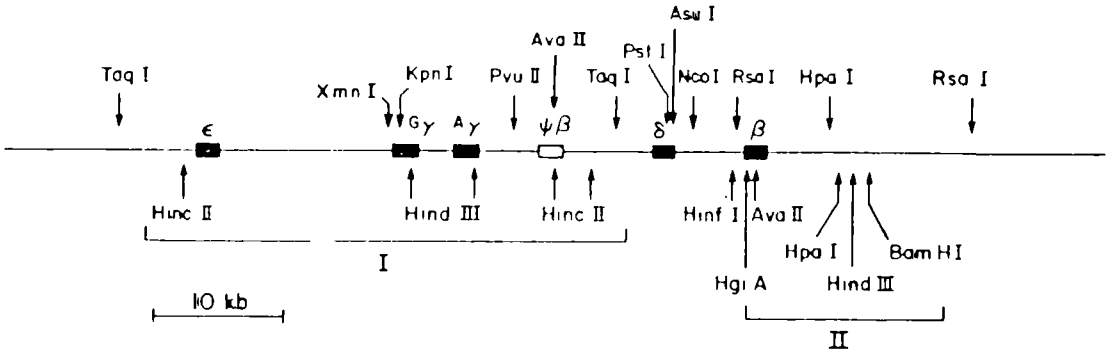


Fig: 1.8 Restriction endonuclease site polymorphisms in human non-α globin gene cluster. The sites shown below the line have been most intensively studied and are commonly used to define chromosomal haplotypes in genetic analyses. Polymorphisms at the sites encompassed in brackets I and II are usually in linkage disequilibrium with one another within each bracketed area, but random recombinations or associations occur between polymorphisms of group I and those of group II (Bunn and Forget 1986).

Many β-thalassaemia mutations can also be identified by restriction enzymes (Thein and Weatherall 1988). RFLP analysis can be used for prenatal diagnosis of thalassaemia by identifying the restriction sites closely linked to the β-globin gene (Old et al, 1986a). A disadvantage of the linkage based diagnosis is that a meiotic recombination between the polymorphic site and the β-globin gene can be a potential source of error (Old et al, 1986b). Requirement of a previously affected or normal child in the family or the grand parents, lack of informativeness of the marker sites in some families (Antonarakis 1989) and the presence of a mutation outside the β-globin gene cluster e.g. in the locus control region (Kazazian et al, 1990) are other disadvantages.

Genomic sequencing:

Sanger et al, (1977) described a method of determining the sequence of unknown DNA. The

method is based on the principle that the inhibitory activity of 2',3'-dideoxythymidine triphosphate (ddTTP) on DNA polymerase-I is incorporated into the growing oligonucleotide chain in the place of thymidylic acid (dT). Because the ddT contains no 3'-hydroxyl group, the chain cannot be extended further, so that termination occurs specifically at positions where dT should be incorporated. If a primer and template are incubated with DNA polymerase in the presence of a mixture of ddTTP and dTTP, as well as the other three deoxyribonucleoside triphosphates, a mixture of fragments, all having the same 5' end with ddT residue at the 3' ends, is obtained. When this mixture is separated by electrophoresis on denaturing polyacrylamide gels the pattern of bands shows the distribution of dTs in the newly synthesized DNA. By using analogous terminators for the other nucleotides in separate incubation reactions and running the samples in parallel on a gel, a pattern of bands is obtained from which the sequence of DNA can be read.

Sequencing of DNA is an extremely useful tool in identification of an unknown gene defect. Currently, sequencing is carried out on fragments of either double stranded or single stranded DNA that are amplified by Polymerase Chain Reaction (Engelke et al, 1988; Rao 1994).

Polymerase chain reaction (PCR):

Polymerase Chain Reaction (PCR) is an in-vitro technique that allows amplification of a specific DNA region (Mullis and Fallona 1987). The amplification of DNA is achieved by using oligonucleotide primers. These are short, single stranded DNA molecules that are complimentary to the ends of a defined sequence of the DNA template. The primers are extended on single stranded denatured DNA (template) by heat stable DNA polymerase in the presence of deoxynucleoside triphosphate (dNTPs) under suitable reaction conditions. This results in the synthesis of a new DNA strand complementary to the template strand. DNA polymerase is used to synthesize new strands of DNA in successive cycles of heat denaturation of the template, annealing of primers by cooling the mixture and primer extension at a temperature suitable for the enzyme. Each cycle of amplification creates new DNA strands that become templates for the next cycles.

PCR products consist of fragment(s) of DNA of size(s) defined by the boundaries of the PCR primers. The simplest and most commonly used method of PCR product analysis is

its electrophoresis on agarose or polyacrylamide gels. DNA is a negatively charged molecule and migrates towards the positive electrode when it is placed in an electrical field. This movement is inversely proportional to the molecular weight of DNA. Once separated by electrophoresis, DNA can be visualized by staining with ethidium bromide or silver nitrate (Newton and Graham 1994).

PCR is an extremely sensitive technique and in about 20 cycles there will be 2^{20} fold amplification. Therefore the commonest source of error in PCR involves contamination at the pre PCR step (Newton and Graham 1994). It is important to include negative controls and reagent blanks with every batch of reactions. Other useful measures to avoid contamination may be the physical isolation of PCR reagents and products, autoclaving solutions, avoiding splashes, use of positive displacement pipettes and aliquoting reagents (Kwok and Higuchi 1989).

The basic technique of PCR has been used in several approaches that identify known and unknown gene defects. These include Amplification Refractory Mutation System (ARMS), dot blot, reverse dot blot, Denaturing Gradient Gel Electrophoresis (DGGE), Single Strand Conformation Polymorphism (SSCP) and chemical cleavage of mismatches (Newton and Graham 1994).

Amplification refractory mutation system (ARMS):

In PCR the yield of reaction is critically dependent on a perfect match between the 3' end of the primer and the template DNA. A primer designed to match the change encountered in a point mutation will only amplify the mutant sequence and similarly the primer for a normal sequence will amplify only the normal sequence and not the mutant sequence (Newton et al, 1989). Kowk et al, (1989) have shown that additional mismatches at position -3 or -4 from the 3' end of the primer can further improve the specificity of the reaction. They also demonstrated that A:G, C:C and to some extent G:G mismatches gave the best results. Studies by Old et al, (1990) and Varawalla et al, (1991) have proven the efficacy of ARMS method in detecting β-thalassaemia mutations.

Denaturing gradient gel electrophoresis (DGGE):

The method is based on the principle that electrophoretic mobility of a double stranded DNA molecule through linearly increasing concentration of denaturing agents is retarded by its denaturation (Myers et al, 1987). As the DNA fragment proceeds through the gradient gel, it will reach a position where the concentration of the denaturing agent equals the melting temperature (T_m) of its lowest melting domain. This results in marked retardation of its electrophoretic mobility. The T_m of a melting domain is dependent on its nucleotide sequence. Therefore, when DNA fragments, that differ by a single nucleotide change in their lowest melting domain, are electrophoresed through denaturing gradient gels, branching and consequent retardation of their mobility will occur at different positions along the gel. This allows their separation. The DGGE can not resolve DNA fragments differing by base changes located in the highest T_m domains. This problem can be overcome by attaching a G-C rich domain (40-45 bases) into the fragment to be analyzed (Myers et al, 1985). The G-C rich domain has a high T_m and it prevents complete denaturation of the DNA molecule. The PCR-DGGE combination is a very useful tool for detection of single neucleotide changes in DNA (Fodde and Losekoot 1994).

Dot blot and reverse dot blot:

The technique of dot blot involves immobilization and denaturation of PCR products to a membrane, such as nitrocellulose or nylon, followed by hybridization with the allele specific oligonucleotides. Using stringent conditions an oligonucleotide that is fully complementary to one allele will hybridize to that allele only. The other allelic variants will, similarly, be hybridized by its specific oligonucleotide probe (Amselem et al, 1988).

The reverse dot blot is similar to dot blot but the process is reversed i.e. allele specific oligonucleotides are immobilized onto a membrane and hybridization is then carried out using a radioactive or biotinylated PCR product. It offers the advantage of simultaneous analysis of several mutations in a single test (Newton and Graham 1994).

2 Pakistan and thalassaemia

Historical background:

The people of Pakistan are made up from all those who, at various times and for various reasons, have come to the land that today is known as Pakistan. The first urban settlements that emerged as Indus Valley Civilization can be traced back in history to beyond 2500 B.C. (Wolpert 1977). However, it was with the arrival of the Aryans from Eurasia between 1500-500 B.C. that the new chapter in the story of Pakistan really begins. Subsequently, the region saw successive invasions by Persians in 530 B.C., Greeks in 330 B.C., Bactrians, Scythians, and Parthians between 185 B.C. and 75 B.C., Kushans in 1st century A.D., Huns in 500 A.D., Arabs in 700 A.D., Turks in 977 A.D., Mongols in 1221 A.D., and Mughals from Central Asia in 1504 A.D. (Rapson 1962).

In spite of the frequent invasions and tribal migrations, mainly from the Central Asia, the vast majority of the population, however, stems from a genetic pool that has its roots in South Asia. The first impression of the Pakistanis likely to be gained by visitors to a big city in Pakistan is of its amazing variety (Halliday 1990).

Population and the ethnic groups:

According to the census conducted in 1981 Pakistan's population was 84.25 million. Presently it is estimated at 137 million and its growth rate is 2.8% (Economic survey 1995-1996).

There are four main ethnic groups (Fig 2.1) i.e. Punjabi, Pathan, Sindhi, and Baluchi who are mainly distributed in the provinces of Punjab, North West Frontier Province (NWFP), Sindh and Baluchistan respectively. In addition, a fifth group of people called Mohajirs, who migrated from various parts of India at the time of creation of Pakistan in 1947, can also be recognized. According to the census (1981) the proportion of the population in various provinces was as follows: Punjab 56.4%, Sindh 22.5%, NWFP 15.8%, and Baluchistan 5.1%.

Fig: 2.1. Four Provinces of Pakistan. The predominant population in each province consists of Pathans in the North West Frontier Province (NWFP), Punjabis in Punjab, Sindhi and Mohajirs in Sindh, and Baluchis in Baluchistan.

Punjabi:

The name Punjab is derived from "punj" meaning five, and "aab" meaning waters. Five major rivers cross the province of Punjab. The inhabitants of Punjab are known as Punjabis. Agriculture, because of an extensive network of rivers and canals, is the most important industry in Punjab. Punjabis are the largest ethnic group numbering approximately 80 million. The racial origin of Punjabis is considered to be from Aryans. However, a mixture from South Indian civilizations is also present (Joshi and Singh 1976).

Pathan:

In the mountainous terrain of the North West Frontier Province (NWFP) the people are mostly Pathans, divided into clans or into families connected with certain villages or districts. The Pathans number approximately 15-20 million. They are perhaps the world's largest tribal society (Quddus 1987). Some of the tribes residing in Pakistan are also distributed over the neighbouring Afghanistan. Many origins have been attributed to Pathans i.e. Aryan, Greek, Persian, Arab and Jewish (Bokhari 1975). What ever their origin may be, they can be distinguished by their sharp features and fair skin.

Sindhi:

The province of Sindh derives its name from the mighty river "Sindh" or "Indus" that is also the reason for the name "India" for the region as a whole. Geographically speaking "Sindh" denotes the lower half of the Indus Valley. Ethnically Sindhi society has been cosmopolitan and its origin ranges from the descendants of the ancient Aryans, Scythians, Arabs, Turks, Persians, Rajputs, and Baluhis (Khan 1980). There are approximately 15 million Sindhis and almost all of them are confined to the province of Sindh.

Baluchi:

Baluchis, the smallest ethnic population numbering about 5 million, are scattered over the largest province of the country accounting for about 42% of its area. Most of Baluchistan has more in common with Western Asia than with the Indian subcontinent. Although there is evidence that the area was inhabited in the stone age (7000-3000 B.C.) the name Baluchistan only came into existence with the arrival of tribes from Iran called "Baluch". There is evidence that this tribal migration took place around 700 B.C. (Bokhari 1975).

Mohajir:

This group is not well defined. It includes mostly individuals and their descendants who migrated from various parts of India during partition of the subcontinent in 1947. These include predominantly Urdu speaking individuals from various parts of Northern India. In addition, a large number of people from Gujrat and Bihar are also included in this group.

Unofficial estimates for the number of Mohajirs are around 15 million. They mostly reside in the province of Sindh.

Caste Biradri and Tribe:

The remnants of the traditional caste system, that took its origin in common with Hinduism in the Aryan era, can also be seen in Pakistani culture. However, social discrimination on the basis of caste, still widespread in India, is not prominent. The Biradri is a community whose members are related either closely or distantly by blood or marriage so that members think of each other as kinsmen (Kolenda 1978). It is much larger as compared to a kinship net work of an individual's kindred in the Western Society. Biradris usually do not have any territorial confinements. There are a number of sizeable Biradris in Pakistan that range in numbers from a few hundred to even millions. Some of the larger Biradris are subdivided in to smaller sub-Biradris. The equivalent of a Biradri amongst the Pathans and Baluchis is a tribe.

The rules for behaviour within the Biradri or a tribe are well defined and agreed upon. The elders usually perform the duties of enforcing the rules, doing justice on disputes and carrying out social planning (Punjabi 1976).

Consanguineous marriages:

A consanguineous marriage is defined as marriage between individuals who have at least one, not too remote, common ancestor. In practice the relationships beyond 2nd cousins are taken as non-consanguineous (Bittles 1994). The offspring of a consanguineous marriage is said to be inbred. The main genetic consequence of consanguinity is an increase in the proportion of homozygotes. Recessive genes, that are unable to express themselves in the heterozygous state, are thus brought to the fore (Bodmer and Cavalli-Sforza 1976).

Marriages in Pakistani culture are usually stable and arranged by family members of the two spouses. Consanguineous marriages are a frequent occurrence. Pakistan is a Muslim country so a marital relationship closer than 1st cousin is prohibited (Al-Quran, Sura Al-Nisa). Studies in the major urban cities of Punjab show that almost 50% of the marriages are between close

relatives (Shami and Zahida 1982; Shami and Iqbal 1983; Shami and Hussain 1984; Shami and Minhas 1984; Shami and Siddiqui 1984; Darr and Modell 1988; Bittles et al, 1993). A study of 9,520 couples in the urban Punjab by Bittles (1994) showed that 50% of the marriages are consanguineous, 34% are between unrelated but Biradri members, and only 16% are between completely unrelated individuals (Table 2.1). Average coefficient of inbreeding, the probability that an individual inherits two genes at a locus that are identical by descent i.e. from a common ancestor, in Punjabis is estimated to be 0.0280 (Bittles 1994).

A study of consanguineous marriages amongst Pathans of Swat (Table 2.1) showed that it was 31% (2^{nd} cousin or closer) in the urban areas and 37% in the rural areas (Wahab and Ahmed 1996). Comparable data from other regions and ethnic groups are lacking. Unpublished reports on consanguineous marriages amongst other ethnic groups are summarized in Table: 2.1. A rural Sindhi sample of 202 couples showed that 73% marriages were consanguineous (Dr. Rafique Memmon, personal communication). In 189 couples from rural Baluchis 87% marriages were consanguineous (Dr. Jaleel Anwar, personal communication). Consanguineous marriages in 120 Mohajir couples, however, were 47.5% (Mr. Mohammad Iqbal personal communication).

Darr and Modell (1988) have shown that consanguineous marriages amongst the British Pakistanis were more common in the present generation of couples as compared to their previous generation. Wahab and Ahmad (1996) also observed similar trend in a study of Pathan couples in Pakistan.

The main effect of parental consanguinity is to increase the risk of recessively inherited disorders in the offspring. It particularly favours the manifestation of rare recessive disorders, because the chance that one carrier will marry another is low unless they are related. When the recessive disorder is common the carrier has a relatively high risk of marrying another however they choose their partner, and the risk is approximately doubled in a first cousin marriage (Modell and Kuliev 1992).

Table: 2.1. Pattern of consanguineous marriages in the five main ethnic groups of Pakistan.

Ethnic Group:	Number Studied:	Urban/ Rural:	DIC:	1st Cousins:	1 ½ Cousins:	2nd Cousins:	Biradri Member:	Unrelated:	Coefficient of inbreeding:	Reference:
Punjabi	9,520	Mostly urban	86 (0.9%)	3,529 (37.1%)	1,115 (11.7%)	54 (0.6%)	3,230 (33.9%)	1,506 (15.8%)	0.0280	Bittles 1994
Pathan	2,037	Mixed	-	448 (22.0%)	108 (5.3%)	139 (6.8%)	169 (8.3%)	1173 (57.6%)	0.0164	Wahab and Ahmad 1996
Sindhi	202	Rural	10 (5.0%)	112 (55.4%)	12 (5.9%)	14 (6.9%)	43 (21.3%)	11 (5.4%)	0.0437	Dr. Rafique Memmon Personal communication.
Baluchi	189	Rural	-	159 (84.1%)	-	5 (2.6%)	?	25 (13.2%)	0.0532	Dr. Jaleel Anwar personal communication.
Mohajir	120	Urban	1 (0.8%)	29 (24.2%)	10 (8.3%)	17 (14.2%)	?	63 (52.5%)	0.0209	Mr. Mohammad Iqbal personal communication

DIC: Double 1st cousin

Haemoglobin disorders in Pakistan:

β-thalassaemia trait:

Stern et al, (1968) reported a 4% carrier rate for β-thalassaemia amongst 129 Pathans. The incidence of thalassaemia in Pakistan remained under reported due to very limited diagnostic facilities (Saleem 1974). The studies on carrier rate of β-thalassaemia in Pakistani population have widely variable results (Table 2.2). Farzana et al, (1975) reported 2.6% incidence in 610 healthy blood donors and medical staff at a hospital in Karachi. In another study of 1224 adults from Karachi, Hashmi and Farzana (1976) found 1.4% carrier rate for β-thalassaemia. A study on 67 Pakistanis in Paris reported an incidence of 3.0% (Coquelete et al, 1983). Latif (1983) found 9.6% carrier rate for β-thalassaemia in a selected sample of 437 individuals from Lahore who had hypochromic and microcytic anaemia. In another study Hameed and Chaudhry (1984) found a carrier rate of 1.6% in 300 healthy adults from Lahore. Ihsanullah et al, (1985) found 6.3% β-thalassaemia carriers amongst 256 individuals in a hospital based study at Karachi. The carrier rate amongst the British Pakistanis is around 6% (Modell and Berdoukas 1984). Dash (1985) has found 3.6% carrier rate for β-thalassaemia in 2000 healthy blood donors from East Punjab in India. In a more recent population based study, conducted at the Armed Forces Institute of Pathology Rawalpindi by Khattak and Saleem (1992a), 5.4% of the 500 individuals were found to be carriers of β-thalassaemia. The later study also identified that the carrier rate in Punjabis was 3.3% and amongst Pathans it was 8.0%. A recent survey of 1000 individuals from Rawalpindi showed 3.9% carrier rate for β-thalassaemia (Hassan et al, 1997).

Variability in results of the studies on thalassaemia carrier rate is mostly due to lack of adequate laboratory facilities, technical difficulties in carrier detection, and inappropriate selection of the target population (Khattak 1987). WHO (1985) has estimated that the overall carrier rate of β-thalassaemia in Pakistan is 5%.

α-thalassaemia:

Little is known about the prevalence of α-thalassaemia in Pakistan. Modell and Berdoukas (1984) have anticipated that the carrier rate for α^+-thalassaemia ($-\alpha/\alpha\alpha$) may be as high as

50% and about 6% of the population may be homozygous for α^+-genotype (-α/-α). Population based data on α-thalassaemia in Pakistan are very scanty (Table: 2.2). Khan and Hayee (1986) screened 320 cord blood samples for red cell indices and Hb-Barts at a hospital in Lahore. They found low MCV and MCH with raised level of Hb-Barts, indicating α-thalassaemia trait, in only 3 (1%) of the new born babies. In another study of Hb-Barts in the cord blood of 500 new born babies in a hospital at Rawalpindi 2.4% had raised level indicating a carrier status for α-thalassaemia (Zuhur-ur-Rehman 1991). Hb-H disease is an uncommon disorder in Pakistan. At the Armed Forces Institute of Pathology, Rawalpindi, each year approximately 150 new cases of β-thalassaemia major are diagnosed and during the same period approximately 3-4 cases of Hb-H disease are also seen (Saleem 1996).

Abnormal haemoglobins:

Lehman and Ager (1961, cited by Stern et al, 1968), were the first to describe sickle haemoglobin in a native of Pakistan. The study of 129 Pathans by Stern et al, (1968) also revealed an individual having Hb-D Punjab. Saleem (1974) reported compound heterozygotes of Hb-S/β-thalassaemia and Hb-E/β-thalassaemia in Punjabi subjects. Hashmi and Farzana (1976) found that 0.9% of 1224 individual from Karachi were carriers for Hb-D (0.65%), Hb-E (0.16%), and Hb-S (0.08%). The largest study of 5000 individuals from Pakistani Armed Forces by Sharma et al, (1976) showed that 39 (0.78%) had an abnormal haemoglobin including Hb-D (0.42%), Hb-E (0.18%), and Hb-S (0.18%). A recent study of 500 healthy individuals from the northern parts of Pakistan showed that 1.2% carried Hb-D trait, and 0.2% were carriers of Hb-E (Khattak and Saleem 1992b).

β-thalassaemia major:

Raheemtoola (1960) was the first to document a case of Cooley's anaemia in Pakistan. Since then relatively few studies have described thalassaemia major (Saleem 1974, Raheemtoola 1981, PMRC 1982, Saleem et al, 1985).

Table: 2.2. Studies on the carrier rate of thalassaemia in Pakistani population.

Disorder:	Ethnic group:	Type of study:	Place:	Subjects:	Methods used:	Carriers:	95% CI	Reference:
β-thalassaemia	Pathan	Population based	Peshawar	129	CEM Electrophoresis	5 (3.9%)	0.56-7.24%	Stern et al, 1968
β-thalassaemia	Mixed, all groups	Population based	Karachi	1224	CEM Electrophoresis	17 (1.4%)	0.73-2.04%	Hashmi and Farzana 1976
β-thalassaemia	?	Population based	Paris	67	CEM Electrophoresis	2 (3.0%)	1.08-7.08%	Coquelet et al, 1983
β-thalassaemia	Mostly Punjabi	Hospital based	Lahore	437	CEM Electrophoresis	42 (9.6%)	6.83-12.36%	Latif 1983
β-thalassaemia	Mostly Punjabi	Population based	Lahore	300	CEM Electrophoresis	5 (1.6%)	0.24-3.16%	Hameed and Chaudhry 1984
β-thalassaemia	?	Hospital based	Karachi	256	CEM Electrophoresis	16 (6.3%)	3.24-9.16%	Ihsanullah et al, 1985
β-thalassaemia	?	?	London	?	?	? (6%)	?	Modell and Berdoukas 1984
β-thalassaemia	Mixed, all groups	Population based	Rawalpindi	500	CEM Electrophoresis	27 (5.4%)	3.42-7.38%	Khattak and Saleem 1992a[@]
β-thalassaemia	Punjabi	Population based	Rawalpindi	245	CEM Electrophoresis	8 (3.3%)	1.14-5.46%	Khattak and Saleem 1992a[@]
β-thalassaemia	Pathan	Population based	Rawalpindi	201	CEM Electrophoresis	16 (8.0%)	4.26-11.74%	Khattak and Saleem 1992a[@]
β-thalassaemia	Mixed	Hospital based	Rawalpindi	1000	CEM Electrophoresis	39 (3.9%)	2.7-5.1%	Hassan et al, 1997
α-thalassaemia	Mostly Punjabi	Population based	Lahore	320	CEM Electrophoresis	3 (0.94%)	0.12-2.0%	Khan and Hayee 1986
α-thalassaemia	Mixed	Population based	Rawalpindi	500	CEM Electrophoresis	12 (2.4%)	1.06-3.74%	Zuhur-ur-Rehman et al, 1991

CEM: Cellulose acetate membrane electrophoresis.
CI: Confidence interval.
[@] Same study (total subjects are 500 that included 245 Punjabi, 201 Pathans, and 54 other groups.

Estimated number of thalassaemia major cases in Pakistan:

The number of cases of a recessive disorder in a population can be calculated from its carrier rate by Hardy Weinberg law which states that $p^2 + q^2 + 2pq = 1$, where p and q are the frequencies of the two alleles under question. Hardy Weinberg equation needs a correction for consanguineous marriages. In a population where mating is not random, the proportion of homozygotes is increased by Fpq. Where F is the inbreeding coefficient (Bodmer and Cavalli-Sforza 1976).

Alwan and Modell (1997) have estimated that in Pakistan with a population of 115 million and 3% annual birth rate, 5% carrier rate for β-thalassaemia, and 50% consanguineous marriages, a little over 3000 β-thalassaemia homozygotes will be born each year (Fig: 2.2).

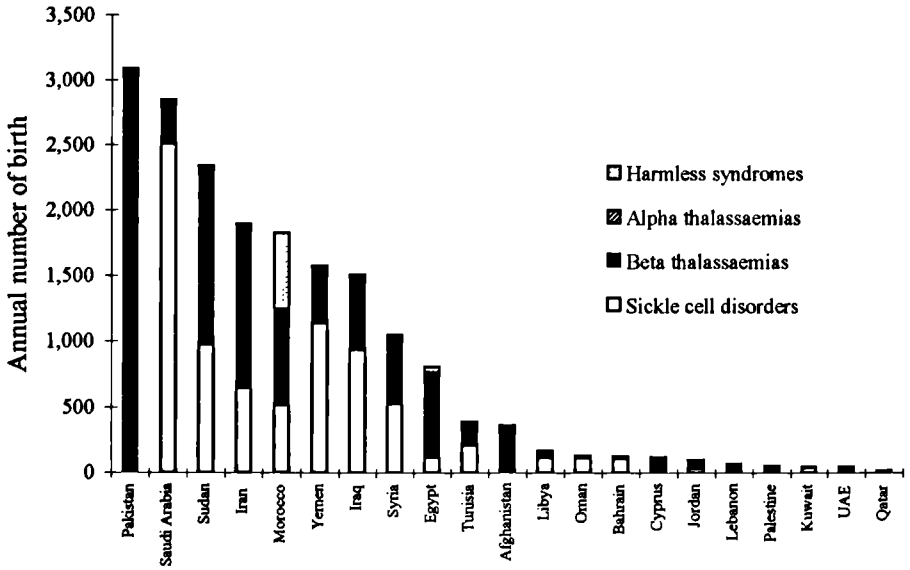


Fig: 2.2. Annual number of infants born with a haemoglobin disorder in the Eastern Mediterranean region (Total=18,600) (Alwan and Modell 1997).

Treatment facilities for thalassaemia:

The Government has paid very little attention to thalassaemia (Saleem 1996). Consequently, there is a lack of awareness amongst the general public and the health professionals. Fatimid Foundation, a charity organization working since 1982, is providing blood transfusion services to over 6000 of its registered patients. This organization runs its centres in Karachi, Lahore,

Multan, and Peshawar. A few other charity organizations in different parts of the country look after an additional 1500 registered cases. Fig: 2.3 summarizes the total number of registered cases in various cities of the country. The registered cases are less than 25% of the estimated 30,000 total in the whole of the country.

Saleem (1996) has comprehensively reviewed thalassaemia at the National level. Retrospective analysis of over 900 cases seen at the Armed Forces Institute of Pathology (AFIP) Rawalpindi showed that the average number of new cases diagnosed per year has steadily increased from 58 in 1984 to 173 in 1995. This is primarily due to increasing awareness amongst the local medical practitioners who refer the cases. The mean age at presentation was 32 months. Most of the children were severely anaemic and had developed moderate to marked hepato-splenomegaly. Most patients come from the rural areas or smaller cities where specialist doctors and adequate facilities for diagnosis are not available. Therefore, a substantial delay in reaching a specialized medical centre for treatment is not unusual.

The quality of treatment that is provided to these patients is far from ideal or even optimum. There is no audit on the treatment protocols being followed at the transfusion centres. Pre-transfusion haemoglobin of patients receiving regular blood transfusions at one of the treatment centres (Fig: 2.4) showed a mean level of 6.5 g/dl with values ranging between 3.0-9.5 g/dl (Saleem 1996). A low pre-transfusion haemoglobin is due to poorly developed transfusion services, inadequate voluntary blood donations, and the lack of awareness amongst the treating physicians and the affected parents about the benefits of hyper-transfusion regimen.

A survey of thalassaemic children receiving treatment at a centre in Pakistan showed that 33% had never received iron chelation, 42% were occasionally getting one injection of desferal at the time of transfusion, 18% were getting desferal at irregular intervals and only 1.5% were on regular desferal therapy (Saleem 1996). The importance of screening of blood for hepatitis B and C has just started to be realized. In a recent study by Bhatti (1995) it was shown that 60% of the multiply transfused thalassaemics are positive for Hepatitis-C. Transmission of HIV, fortunately, is not a significant problem at least in the northern areas of Pakistan (Tariq and Hussain 1995).

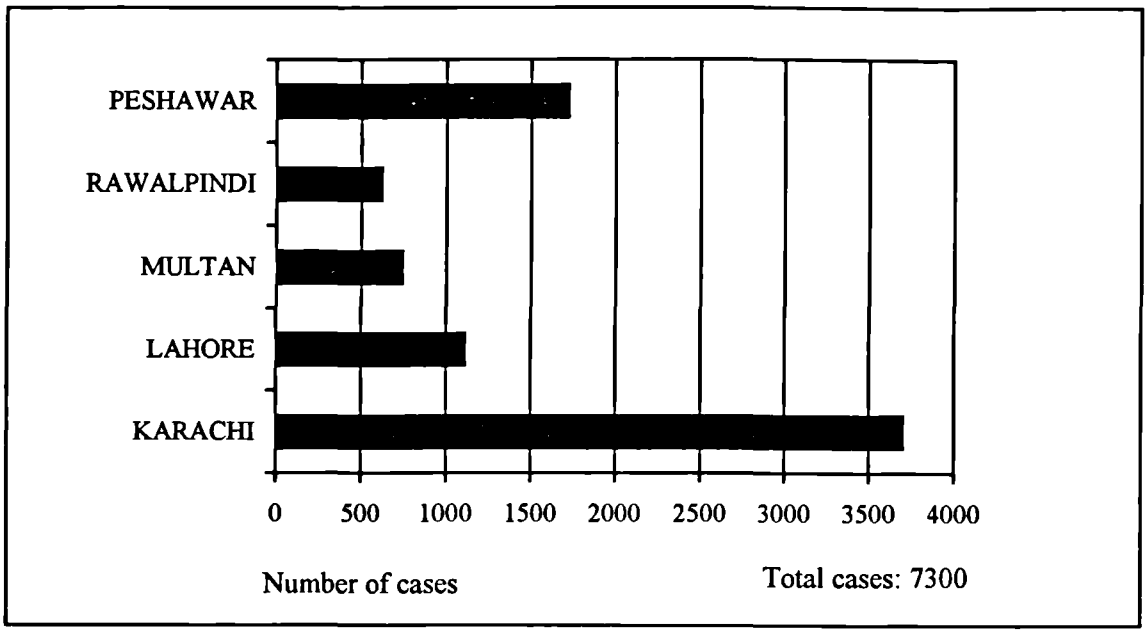


Fig: 2.3. The number of registered cases of β-thalassaemia major in various cities of Pakistan (Saleem 1996).

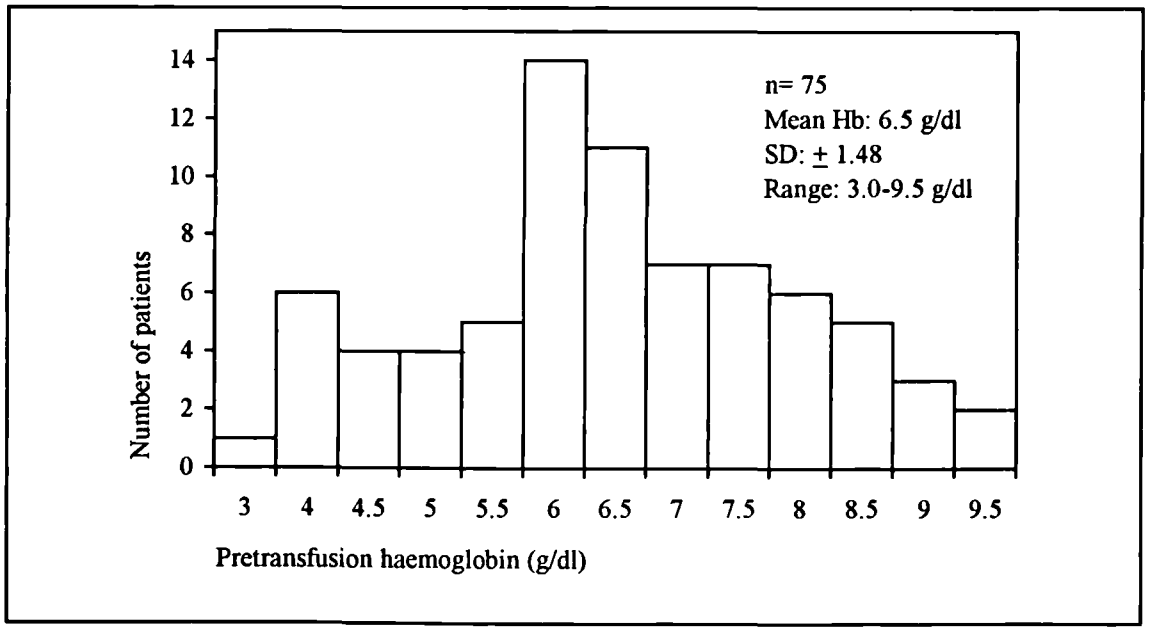


Fig: 2.4. Frequency distribution of pretransfusion haemoglobin in 75 patients of β-thalassaemia major at one of the treatment centres in Pakistan (Saleem 1996).

Poor management of thalassaemics is reflected by an average survival of 10.5 years (Fig 2.5) in 76 deceased children getting treatment at one of the centres in Pakistan (Saleem 1996). The average survival of 10.5 years is for patients who receive treatment, whereas at least 2/3rd of thalassaemics in Pakistan do not have an access to a treatment centre and they probably die during very early childhood. As a result of poor survival and poor quality of life the affected families experience tremendous psycho-social burden.

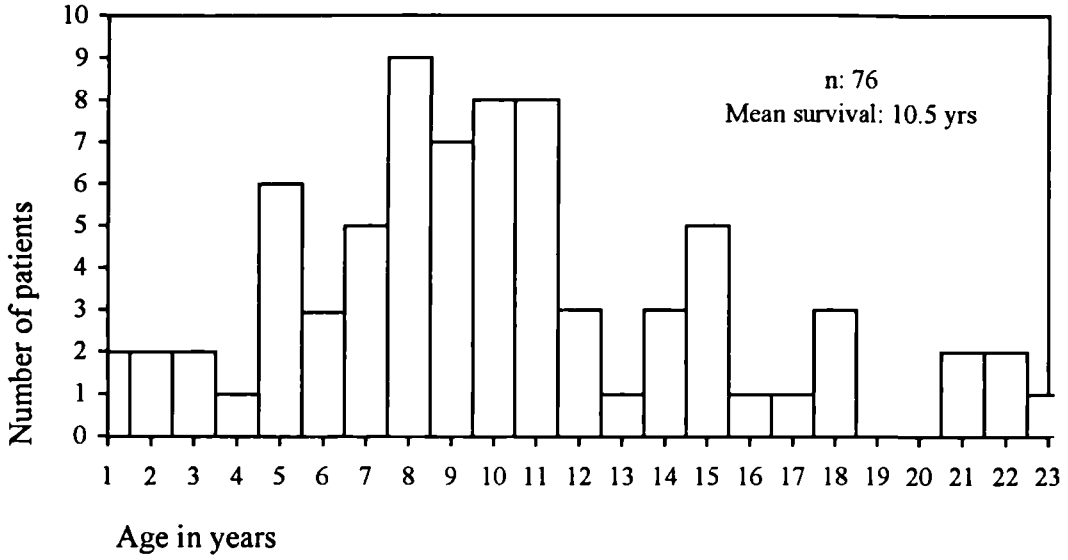


Fig: 2.5. Age distribution at the time of death in 76 patients with thalassaemia major who received blood transfusions at one centre in Pakistan (Saleem 1996).

Thalassaemia and consanguineous marriage:

When a recessive disorder is common, 5% β-thalassaemia carriers in Pakistan for example, the carriers have a relatively high risk of marrying another carrier however they choose their partner, and the risk is approximately doubled in a first cousin marriage (Modell and Kuliev 1992). Saleem (1996) in a retrospective analysis of 602 patients of β-thalassaemia major observed that the parents of 357 (59%) patients were 1st cousins, 145 (24%) had more distant related parents, and only 100 (17%) had completely unrelated parents.

Prevention of thalassaemia in Pakistan:

There is very little awareness about the concept of prevention of thalassaemia amongst the health professionals and the general public in Pakistan. Genetic counselling is available to only a fraction of the couples attending various treatment centres. Carrier screening although available at many private laboratories is not widely used. The main reason for the under utilization of the screening facilities is the lack of awareness amongst the health professionals dealing with thalassaemic families and the high cost. The quality of work at the private laboratories is also dubious. There is a general lack of counselling of the carriers. Inadequate counselling of the affected families is also due to the non-availability of prenatal diagnostic facilities within the country.

Alwan and Modell (1997) have calculated (Fig: 2.6) that the number of pregnancies at risk of a clinically significant haemoglobin disorder in Pakistan is in excess of 12000 every year. These figures are the highest for β-thalassaemia in the Eastern Mediterranean Region. Screening of such large number of pregnancies would require tremendous financial and administrative resources. The first step would be to identify a pregnancy at risk. In a Pakistani setting, where the majority of the pregnancies escape medical attention, it will be a challenging task. The next problem would be to establish centre(s) where facilities for prenatal diagnosis are available. This would also involve in depth studies of β-thalassaemia mutations in all ethnic groups of Pakistan and a cost-effective strategy for carrying out prenatal diagnosis. Varawalla et al, (1991a; 1991b), have studied the mutations causing β-thalassaemia in 167 subjects from the northern region of Pakistan. They have identified seven different mutations in the study population (Fig: 2.7).

Another important issue that would determine the success of a thalassaemia prevention programme in Pakistan would be the attitude of the affected couples towards prenatal diagnosis and termination of pregnancy. In the Mediterranean, extensive data now exists about the relative acceptability of the options open to the at risk couples. The absence of equivalent objective information for the predominantly Muslim countries of the Eastern Mediterranean Region, including Pakistan, introduces considerable uncertainty about the success of a prevention programme (Alwan and Modell 1997). Some information about the attitude of the

British Pakistanis towards prenatal diagnosis and termination of pregnancy is available. The British Pakistanis have a greater difficulty requesting prenatal diagnosis than most other ethnic groups in the UK. However, when couples are adequately counselled, they often make use of prenatal diagnosis, although they have particular problems with late termination of pregnancy (Petrou 1994).

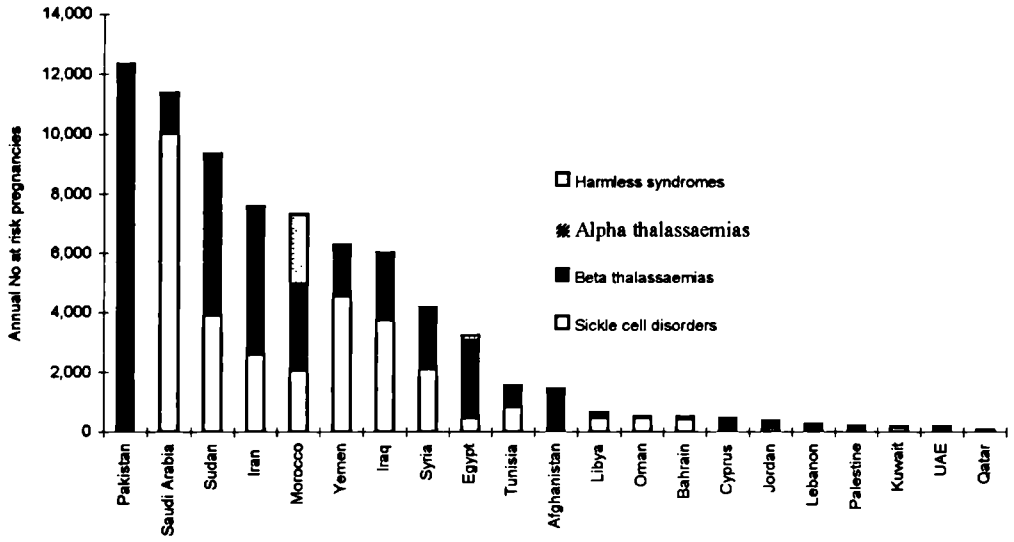


Fig: 2.6. Annual pregnancies at risk of a haemoglobin disorder in the Eastern Mediterranean region (Total=74,250) (Alwan and Modell 1997).

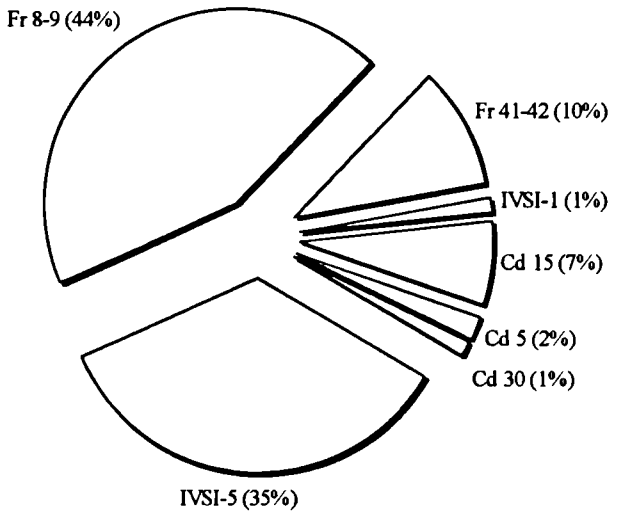


Fig: 2.7. β-thalassaemia mutations in 167 individuals from the Northern region of Pakistan (Varawalla et al, 1991a and b).

3 Materials and methods

Sequence and the time frame of the work:

The work in this study was done at the Department of Haematology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan and the Perinatal Centre, Department of Obstetrics and Gynaecology, University College Medical School, London. Table 3.1 gives a summary of the events, in a chronological order.

Haematological Methods:

Red cell indices:

The basic haematological parameters evaluated include, Haemoglobin (Hb) estimation, Total Red Blood Cell Count (TRBC), Mean Cell Volume (MCV), and Mean Cell Haemoglobin (MCH). These parameters were measured on blood collected in EDTA. An automated cell counter Sysmex K-1000 was used. However, Coulter S880 was also available as a backup support. Both instruments work on the principle of the electronic resistance method (Dacie and Lewis 1991). The instruments were calibrated according to the manufacturers instructions using the high, low and normal whole blood controls. Once calibrated, the performance of the instruments was regularly checked after every 20 samples by running a control.

Haemoglobin electrophoresis and Hb-A_2 estimation:

Blood collected in EDTA was thrice washed in normal saline (9.0 g/L NaCl). The washed red cells were lysed by adding two volumes of water and one volume of carbon tetrachloride (CCl_4). Clear haemolysate was separated after shaking and centrifugation at 1200 g for 10-20 minutes (Dacie and Lewis 1991). In a simple alternative method haemolysate was prepared by adding an equal volume of 1% saponin to the washed red cells. The final concentration of the haemolysate was adjusted to around 10 g/dl.

Table: 3.1. A chronological summary of the work carried out in the study.

Sr. No:	Event:	Place:	Period:
1.	Collection /extraction of DNA samples from the northern areas of Pakistan.	AFIP, Rawalpindi.	June 1992 to July 1993.
2.	Gene analysis of the DNA samples by ARMS method.	Perinatal Centre, UCL London.	July 1993 to January 1994.
3.	Establishment of facilities for gene analysis by PCR at AFIP Rawalpindi.	AFIP, Rawalpindi.	February 1994.
4.	Collection/extraction of DNA samples from southern areas of Pakistan and their mutation analysis by ARMS.	AFIP, Rawalpindi.	February 1994 to May 1994.
5.	Start of prenatal diagnostic service for thalassaemia in Pakistan.	AFIP, Rawalpindi.	May 1994.
6.	Thalassaemia Carrier screening in Punjabis and Pathans.	AFIP, Rawalpindi.	May 1994 to August 1994.
7.	Thalassaemia carrier screening in pregnant females.	AFIP, Rawalpindi.	August 1994 to September 1994.
8.	Thalassaemia carrier screening and collection of DNA samples in two large thalassaemic families.	AFIP, Rawalpindi.	October 1994 to December 1994.
9.	Gene analysis by DGGE.	Perinatal Centre, UCL London.	July 1995.
10.	Genomic sequencing of uncharacterized alleles.	Perinatal Centre, UCL London.	August 1995 to September 1995.
11.	Short Tandem Repeat (STR) analysis on DNA samples from the two families and controls.	Perinatal Centre, UCL London.	October 1995 to December 1995.
12.	Thalassaemia carrier screening on the extended families.	AFIP, Rawalpindi.	January 1995 to February 1995
13.	Thalassaemia carrier screening in Baluchi, Sindhi and Mohajirs.	AFIP, Rawalpindi.	March 1995
14.	Gene analysis for α-thalassaemia.	AFIP, Rawalpindi.	April 1995
15.	Assessment of the response of thalassaemic families to the availability of prenatal diagnosis.	Fatimid Thalassaemia Centre, Lahore.	May 1995

Haemoglobin electrophoresis was carried out on cellulose acetate membranes at alkaline pH (Dacie and Lewis 1991). At alkaline pH haemoglobin is a negatively charged protein and in an electrical field moves towards the anode (+). Most structural forms of haemoglobin can be separated due to a difference in the amount of charge on their surface.

The electrophoresis was carried out in a buffer containing Tris/EDTA/borate at pH 8.5. The cellulose acetate strips (Shandon UK) were allowed to soak in the buffer for at least 10 minutes. The haemolysate was applied to the cathode end of the strip. Electrophoresis was carried out at 200 V for 30 minutes or until adequate separation was achieved. The strips were stained in Ponceau-S and were subsequently dried at 37°C for 30 minutes. With each batch of samples haemolysates prepared from cord blood and known cases of β-thalassaemia trait were included as controls.

Hb-A_2 was separated electrophoretically on cellulose acetate at pH 8.9 after applying 5-10µl of the haemolysate. The bands of Hb-A and Hb-A_2 were carefully cut and placed in 15 ml and 1.5 ml respectively of the buffer. Elution of the bands was allowed for 20-30 minutes. Absorbance of each fraction was measured at 413 nm against the buffer as blank. Percentage of Hb-A_2 was calculated as follows:

$$\%\text{Hb-}A_2 = \frac{A^{413}\ \text{Hb-}A_2}{(10 \times A^{413}\ \text{Hb-A}) + A^{413}\ \text{Hb-}A_2} \times 100$$

Microcolumn chromatography kits (Bio-rad, USA) were used on some samples to crosscheck the results of Hb-A_2. Commercial controls of Hb-A_2 (Bio-rad, USA) were used to check the quality control.

Strategy for carrier screening:

β-thalassaemia:

Haematological parameters including Hb, TRBC, MCV and MCH were recorded. Hb-A_2 estimation was done on all those who had MCV ≤75 fl or MCH ≤25pg (Modell and Berdoukas 1984). Hb-A_2 ≥3.5% confirmed the diagnosis of β-thalassaemia trait (Steinberg and Adams III 1991). In subjects who had Hb-A_2 in the borderline range (3-

3.4%) or who had Hb <9g/dl and Hb-A_2 in the normal range, multiplex ARMS PCR was carried out to screen for the thalassaemia mutations (Cao et al, 1994).

Abnormal haemoglobins:

Screening for abnormal haemoglobins by electrophoresis was possible only in cases that had MCV ≤75 fl or MCH ≤25pg.

α-thalassaemia:

α-thalassaemia screening was done by the PCR based detection of $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions (Baysal and Huisman 1994).

Gene analysis:

DNA extraction:

For gene analysis 5ml of blood was collected in EDTA. The red cells were lysed by 1% solution of triton X-100 and the white cells were lysed by 0.5 ml of 2% SDS solution containing 50 mM Tris and 20 mM Na_2EDTA (pH 8.0). Protein digestion was done with 250 μg Proteinase-K (Sigma, USA) at 37°C overnight or at 56°C for 2 hours. DNA extraction was done by phenol chloroform method (Maniatis 1989). The extraction was done in 1.5 ml Eppendorf tube by adding 250μl each of buffered phenol containing 0.1% 8-hydroxyquinolone (pH 8.0) and chloroform. Phenol chloroform extraction was done twice and was then followed by one wash in chloroform. Final precipitation of DNA was carried out in 70% ethanol. The extracted DNA was dried and re-dissolved in water to yield a final concentration of approximately 500ng/μl. The DNA samples were stored at -20°C until analysis.

Mutation analysis:

Strategy for mutation analysis:

The samples were first screened by the Amplification Refractory Mutation System (ARMS) for 15 β-thalassaemia mutations previously reported in Pakistani and Indian subjects (Varawalla et al, 1991a and 1991b). Those who were not positive for these mutations were screened by Denaturing Gradient Gel Electrophoresis (DGGE) as this

identifies the region of the gene where the mutation may be present (Cai and Kan 1990). The region of β-globin gene that was found by DGGE to have a mutation was sequenced by the di-deoxy chain termination method (Sanger et al, 1977). In samples where a mutation was not found by ARMS or an abnormal fragment by DGGE, had the entire β-globin gene sequenced with its immediate 5' and 3' flanking sequences.

Amplification Refractory Mutation System (ARMS):

The β-globin mutations screened for, and the sequence of the ARMS primers used is presented in Table: 3.2. The primers were in use for prenatal diagnosis of β-thalassaemia at the Perinatal Centre, UCH, London. Most of the primers were used in combination with an upstream (5') primer (No 3 in Table: 3.2). However, for Cd 15 (G-A) and Cap+1 (A-C) a downstream (3') primer (No 4 in Table: 3.2) was used. Another set of primers (No 1 and 2 in Table: 3.2) was used to amplify an 861 bp fragment of the distal part of the β-globin gene, to serve as an internal control for the PCR. The same set of primers was also used to screen for the 619 bp deletion, which when present results in amplification of a 242 bp fragment instead of the usual 861 bp fragment. Patients with homozygous β-thalassaemia who were positive for a mutation were then tested for the presence of the normal allele to test if they were homozygous for the mutation or not. Primers for the normal sequence (Table: 3.3) were used to identify the normal allele. Subjects with the normal allele were then tested for the presence of other mutations.

PCR conditions for ARMS:

The PCR for ARMS was carried out in a 25μl reaction mixture containing 5 pM of each primer, 0.5 units of Taq polymerase (Advanced Biotechnologies, UK), 30 μM of each dNTP (Advanced Biotechnologies, UK), 10 mM Tris HCl (pH 8.3), 50 mM KCL, 1.5 mM $MgCl_2$, 100 mg/ml gelatin (Sigma, UK), and 0.3-0.5 μg of genomic DNA. Primers were synthesized by Oswell UK, and Pharmacia Biotech, UK. Thermal cycling was carried out in an automated DNA thermal cycler (TC-480) from Perkin Elmer, USA. The regimen consisted of 25 cycles each consisting of denaturation at 94°C for 1 minute, primer annealing at 65°C for 1 minute, and DNA extension at 72°C for 1½ minutes. In the final cycle, the extension reaction was prolonged for another 3 minutes.

Table: 3.2. The mutations screened for and the sequence of the ARMS primers used.

Mutation	Primer	Used with	Size
IVSI-5 (G-C)	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG	3	285
Fr 8-9 (+G)	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC	3	215
IVSI-1 (G-T)	TTA AAC CTG TCT TGT AAC CTT GAT ACG AAA	3	281
Fr 41-42 (-TTCT)	GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT	3	439
Del 619 bp	CAA TGT ATC ATG CCT CTT TGC ACC	2	242
Cd 15 (G-A)	TGA GGA GAA GTC TGC CGT TAC TGC CCA GTA	4	500
Cd 5 (-CT)	ACA GGG CAG TAA CGG CAG ACT TCT CCG CGA	3	205
Cd 30 (G-C)	TAA ACC TGT CTT GTA ACC TTG ATA CCT ACG	3	280
Cd 30 (G-A)	TAA ACC TGT CTT GTA ACC TTG ATA CCT ACT	3	280
Fr 16 (-C)	TCA CCA CCA ACT TCA TCC ACG TTC ACG TTC	3	238
IVSII-1 (G-A)	AAG AAA ACA TCA AGG GTC CCA TAG ACT GAT	3	634
Cd 26 (G-T)(Hb-E)	TAA CCT TGA TAC CAA CCT GCC CAG GGC GTT	3	278
Cap +1 (A-C)	ATA AGT CAG GGC AGA GCC ATC TAT TGG TTC	4	567
Fr 47-48 (+ATCT)	ATA ACA GCA TCA GGA GTG GAC AGA TAG ATC	3	467
1VS1-25 (25b del)	CTC TGG GTC CAA GGG TAG ACC ACC AGC ATA	3	354
Hb-S	CCC ACA GGG CAG TAA CGG CAG ACT TCT GCA	3	207
1. CONTROL	CAA TGT ATC ATG CCT CTT TGC ACC		
2. CONTROL	GAG TCA AGG CTG AGA GAT GCA GGA		
3. COMMON	ACC TCA CCC TGT GGA GCC AC		
4. COMMON	CCC CTT CCT ATG ACA TGA ACT TAA		

Table: 3.3. Primers used for identifying the normal alleles of the respective mutations.

Allele:	Primer for the normal sequence:	Used with:	Frag Size:
IVSI-5	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC	3	285
Fr 8-9	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACT	3	214
IVSI-1	GAT GAA GTT GGT GGT GAG GCC CTG GGT AGG	4	450
Fr 41-42	GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA	3	443
Cd 15	TGA GGA GAA GTC TGC CGT TAC TGC CCA GTG	4	500
Cd 5	ACA GGG CAG TAA CGG CAG ACT TCT CCG CAG	3	207
Cd 30	TAA ACC TGT CTT GTA ACC TTG ATA CCT ACC	3	280
IVSII-1	AAG AAA ACA TCA AGG GTC CCA TAG ACT GAC	3	634
Fr 16	TCA CCA CCA ACT TCA TCC ACG TTC ACG TTG	3	238
1. CONTROL	CAA TGT ATC ATG CCT CTT TGC ACC		
2. CONTROL	GAG TCA AGG CTG AGA GAT GCA GGA		
3. COMMON	ACC TCA CCC TGT GGA GCC AC		
4. COMMON	CCC CTT CCT ATG ACA TGA ACT TAA		

Electrophoresis of ARMS products:

At the end of PCR 20µl of the amplified product was mixed with 5µl of the loading dye (0.25% bromophenol blue in 40% sucrose). Electrophoresis was carried out in 4% NuSieve Agarose gel (FMC Corporation, USA) at 150 V for 1 hour. The gels were stained in ethidium bromide (250µg/100ml) for 30 minutes and were visualized and photographed under 302 nm UV light.

Multiplex ARMS PCR:

A multiplex PCR for ARMS (Newton and Graham 1994) was carried out under the standard PCR conditions but instead of using one ARMS primer specific for one mutation, several primers were mixed. The combination of ARMS primers used for screening in multiplex PCR is presented in Table: 3.4. The three multiplex primers, AD-1, AD-2 and AD-3, were prepared as stock solutions containing 5pmol/µl each of all the primers for the mutant alleles. In addition, the two control primers and the respective common primer were also added to the stock mixture. In each ARMS reaction 1µl of the stock primers was used.

The amplified products of all mutations were sufficiently different for resolution by polyacrylamide gel electrophoresis. However, the size of the fragments generated by IVSI-1 and IVSI-5 differed by 5 bp and therefore was difficult to resolve. Similarly there was no difference between the fragments of Cd30 G-C and G-A and IVSI-1. The problem of differentiating IVSI-1 and IVSI-5 was overcome by adding IVSI-1 primer to AD-1 and AD-2 groups. IVSI-5 resulted in amplification with AD-1, but IVSI-1 caused amplification with AD-1 and AD-2. Amplification with AD-2 but not AD-1 indicated Cd30. The difference between the two mutations in Cd30 was only of academic interest because the same normal primer was required to differentiate between the homozygotes and heterozygotes. AD-3 primer combination included Cd15 and Cap+1 as their common primer was different from the one used with other mutations (Table 3.2).

An allelic ladder for the respective mutations was prepared by pooling together the PCR products of separately amplified reactions for various mutations. Each fragment was

amplified in 50μl reaction mixture under standard ARMS conditions. The individually amplified products were pooled and were kept frozen in aliquots. 5μl of the pooled product was used in all polyacrylamide gel electrophoresis runs.

Table: 3.4. Combination of various mutations screened by multiplex ARMS PCR.

Primer ID:	**Mutations pooled:**	**Amplified product size:**
AD-1	Fr 8-9 (+G)	215 bp
	IVSI-5 (G-C)	285 bp
	Fr 41-42 (-TTCT)	439 bp
	IVSI-1 (G-T)	280 bp
	Del 619bp	242 bp
AD-2	Cd 5 (-CT)	205 bp
	Fr 16 (-C)	238 bp
	IVSI-1 (G-T)	280 bp
	Cd 30 (G-C)	280 bp
	Cd 30 (G-A)	280 bp
	IVSII-1 (G-A)	634 bp
AD-3	Cd 15 (G-A)	500 bp
	Cap+1 (A-C)	567 bp

Electrophoresis of multiplex ARMS products:

The multiplex ARMS amplified products were resolved by non-denaturing polyacrylamide gel electrophoresis on Mini-Protean electrophoresis apparatus (Bio-rad, USA). 2 μl of the amplified product was loaded on 6% non-denaturing polyacrylamide gels measuring 1mm X 10cm X 10cm. Electrophoresis was carried out at 150V for 40 minutes. The gels were stained by silver nitrate. The details of gel preparation and silver staining are described in the subsequent sections.

Denaturing Gradient Gel Electrophoresis (DGGE):

β-globin gene was amplified in several overlapping fragments as shown in Fig: 3.1. Table: 3.5 shows the sequence of the primers used for amplification of various fragments of the β-gene. The primers were in use at the Perinatal Centre, UCH, London.

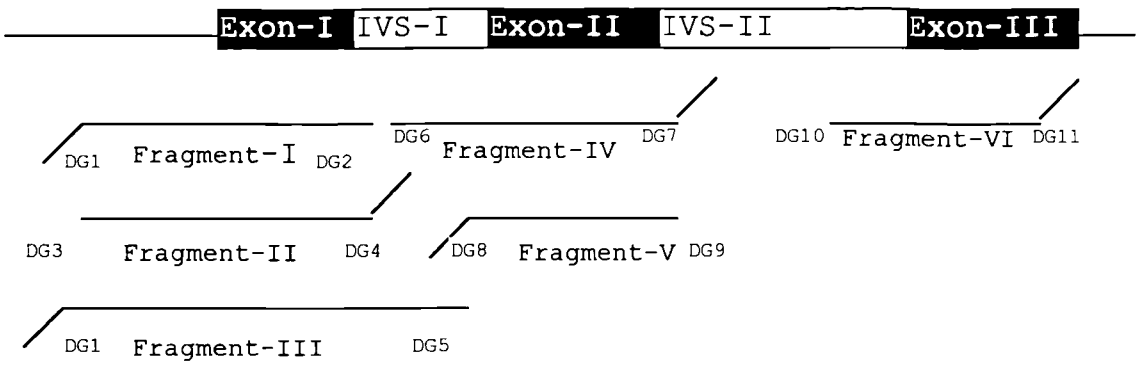


Fig: 3.1. Fragments of β-globin gene that were amplified for DGGE.

PCR conditions for DGGE:

PCR for DGGE was carried out in a 50μl reaction mixture containing 10 pM of each primer, 1.0 unit of Taq polymerase (Perkin Elmer, UK), 200 μM of each dNTP (Boehringer Manheim), 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 mg/ml gelatin and 0.3-0.5 μg of genomic DNA. Thermal cycling consisted of initial denaturation for 5 minutes at 95°C and then 40 cycles each of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, and DNA extension at 70°C for 1 minute. In the final cycle the extension was prolonged to 3 minutes. 10μl of the amplified product was run on 2% agarose gel to determine the quality of amplification.

Preparation of denaturing gels:

The following stock solutions were prepared:

1. 40% acrylamide (Molecular Biology grade. BDH, UK) and 1.07% bisacrylamide (Sigma Chemicals, UK).
2. 20 X gel running buffer (TAE pH 7.4) containing 800 mM Tris base, 400 mM Sodium acetate, and 20 mM EDTA (BDH, UK).
3. 80% denaturant stock solution containing 7% acrylamide, 32% formamide (BDH, UK), 5.6 M Urea (BDH, UK) and 1 X TAE buffer (pH 7.4).
4. 0% denaturant stock solution containing 7% acrylamide, and 1 X TAE buffer (pH 7.4).

Formamide was deionized before use by gently stirring with Dowex AG50W (20-40) mesh Mixed bed resin (Bio-rad, UK). The stock solutions of 0% and 80% denaturent were used to prepare the gradients of varying strengths. Gels measuring 20 X 16 cm and 1.5 mm in thickness were poured by using a gradient mixer (Hoefer Scientific, USA). Ammonium persulphate (100µl of 10% stock solution) and 10µl of TEMED (Sigma Chemical, UK) were used as gel polymerization catalysts.

Formamide urea gradient varied for different fragments of the β-globin gene. The optimal gradient was determined by trial and error. The fragments-I, II and III were run on a denaturant gradient of 42% and 72%. The fragment-IV and V were run on 50%-80% gradient and the fragment-VI was run on 25%-50% gradient.

Table: 3.5. DGGE primers for amplification of various fragments of β-globin gene.

Frag-ment:	ID	Location:	Sequence:	Size:
I:	DG1	-123 to -104	5' 40[GC] CTG TCA TCA CTT AGA CCT CA	286 bp
	DG2	Exon-I Cd 24-17	5' CAA CTT CAT CCA CGT TCA CC	
II:	DG3	-129 to -104	5' GTA CGG CTG TCA TCA CTT AGA CCT CA	297 bp
	DG4	Exon-I Cd 24-17	5' 45[GC] CAA CTT CAT CCA CGT TCA CC	
III:	DG1	-123 to -104	5' 40[GC] CTG TCA TCA CTT AGA CCT CA	424 bp
	DG5	IVSI-119 to 101	5' AAA ATA GAC CAA TAG GCA G	
IV:	DG6	IVSI-29 to 48	5' AAG GAG ACC AAT AGA AAC TG	409 bp
	DG7	IVSII-30 to 11	5' 45[GC] AGA AAA CAT CAA GGG TCC CA	
V:	DG8	IVSI-101 to 120	5' 40[GC] CTG CCT ATT GGT CTA TTT TC	321 bp
	DG9	IVSII-30 to 11	5' AGA AAA CAT CAA GGG TCC CA	
VI:	DG10	IVSII 588 to 607	5' ATG ATA CAA TGT ATC ATG CC	339 bp
	DG11	Exon-III Cd 132-125	5' 45[GC] TCT GAT AGG CAG CCT GCA CT	

GC Clamp
45[GC] GCGGGCGGGGCGGGGGCACGGGGGGCGCGGCGGGCGGGGCGGGGG
40[GC] GCGGGCGGGGCGGGGGCACGGGGGGCGCGGCGGGCGGGGC

Running conditions for DGGE:

Electrophoresis was carried out on SE600 vertical polyacrylamide gel running system (Hoefer Scientific, USA). The original buffer bath was replaced by a specially designed acrylic container designed to hold the gel assembly as well as the heating and the buffer circulation

device. Once the gradient gel had polymerized, the comb was carefully removed and the wells were washed with 1 X gel running buffer. Each well was loaded with 7μl of amplified product mixed with 6μl of loading dye. The quantity of amplified product was increased to 8-10μl depending on the quality of amplification. Once loaded, the gel was removed from the casting assembly and the upper buffer reservoir of the system was secured in place. The Hoefer SE600 accommodates two gels at a time. The whole assembly was placed in the main buffer compartment and the heating and buffer circulating device was turned on.

Electrophoresis was carried out at 50V for 16 hours. Throughout the procedure buffer temperature was maintained at 60°C. The gels were then removed and the glass plates carefully separated. The gel was stained in ethidium bromide (250μg/100 ml) solution for 30 minutes. It was photographed at 302 nm UV light.

Genomic sequencing:

The primers used for amplifying the β-globin gene and the internal sequencing primers are shown in Table: 3.6.

PCR amplification for sequencing:

The whole of β-globin gene (-308 bp from the Cap site to +475 bp from the termination site) was amplified by a set of primers in which the 5' primer was biotinylated (Table: 3.6). PCR was carried out in 100 μl reaction mixture containing 20 pM each of the two primers and 2 units of Taq polymerase (Perkin Elmer, UK). The thermal cycling consisted of 1st cycle of denaturation at 94°C for 4 minutes, primer annealing at 56°C for 3 minutes and extension at 72°C for 2 minutes. In the subsequent 35 cycles the timing of denaturation and primer annealing were reduced to 1 minute and 2 minutes respectively. In the last cycle the extension reaction was prolonged to 8 minutes. The quality of amplification was checked by running 10μl of the product on 2% agarose gel.

Preperation of single stranded template DNA:

DNA amplified with biotinylated primer was used for preparation of single-stranded template. Streptavidin coated Dynabeads (M-280, Dynal UK) and a magnetic particle concentrator

(MPC-E, Dynal UK) was used to separate and concentrate the single stranded DNA (Hultman et al, 1989; Thein and Hinton 1991). The dynabeads were washed in TES (10 mM Tris-HCl, 1 mM EDTA, and 100 mM NaCl at pH 8.0). Approximately 80µl of the amplified product was mixed (without vortexing) with 50µl of dynabeads. It was left on the bench for 5 minutes followed by denaturation with freshly prepared 0.15 M NaOH. The denatured single stranded template was washed in TES and concentrated to yield a final volume of 20µl.

Preparation of double stranded template DNA:

For the majority of the sequencing reactions double stranded DNA template was used. The amplified double stranded DNA contains excess of unused amplification primers, dNTPS and some extraneous single stranded DNA produced by PCR. Two hydrolytic enzymes i.e. Shrimp alkaline phosphatase, and Exonuclease-I can remove the unwanted accompaniments. The two enzymes are available in a kit (United States Biochemical, USA). In the protocol, 5µl of the amplified product was treated with 2 units of Shrimp alkaline phosphatase and 10 units of Exonuclease-I. The reaction mixture was incubated at 37°C for 15 minutes in a thermal cycler followed by an additional 15 minutes at 80°C to inactivate of the two enzymes.

Labelling reaction:

The labelling reaction was done by the Sequenase version 2.0 sequencing kit (United States Biochemical, USA). The method described by Tabor and Richardson (1990), based on di-deoxy chain termination method was used. The enzyme treated PCR product was mixed with 5 pM of internal sequencing primer (Table: 3.6) and water to yield a final volume of 10µl. It was denatured at 100°C in a thermal cycler for 2-3 minutes. It was then cooled as quickly as possible by directly submerging the reaction vial in crushed ice for 5 minutes.

The following were added to the ice cold denatured DNA: 1µl of 0.1 M dithiothreitol (DTT), 2µl of 1:5 diluted labelling mix, 0.5µl of ^{35}S dATP (5 mCi) (Amersham UK), 2µl reaction buffer, and 2µl of diluted Sequenase DNA polymerase. The reaction mixture was incubated at room temperature for 5 minutes. In the mean while four tubes containing 2.5 µl termination analogues (G, A, T and C) were warmed at 37°C for 1 minute. 3.5µl of the labelling reaction was added to the side of each of the four termination analogue tubes avoiding direct contact

between the two. The reaction tubes were quickly centrifuged for a few seconds to allow mixing of the labelling reaction and the termination analogues. The tubes were incubated for 5-10 minutes at 37°C. The reaction was stopped by adding 4µl of stop solution (95% formamide, 0.05% Xylene Cyanol and 0.05% Bromophenol blue). The reaction tubes were frozen until electrophoresis.

Table: 3.6. The primers and their sequence used in sequencing the β-globin gene.

Primers used for amplification of β-globin gene:

5'(Biotinylated) -307 from Cap site:	5' CGA TCT TCA ATA TGC TTA CCA AG
3' +475 from termination site:	5' GAG TCA AGG CTG AGA GAT GCA GGA

Internal sequencing primers:

ID:	Location:	Sequence:
SE1	Cd 16	5' CCC ACA GGG CAG TAA CGG CAG ACT TCT CCT
SE2	Cd 33	5' ACC AGC AGC CTA AGG GTG GGA AAA TAG ACC
SE3	IVSII-24	5' ACA TCA AGG GTC CCA TAG AC
SE4	IVSII-142	5' GAT CCT GAG ACT TCC ACA CT
SE5	IVSII-332	5' ATG TAC TAG GCA GAC TGT GT
SE6	IVSII-742	5' GGA TTG TAG CTG CTA TTA GC
SE7	Cd 142	5' CCA GGG CAT TAG CCA CAC CA
SE8	+196 from term site	5' AAT GCA CTG ACC TCC CAC ATT CC

Preparation of sequencing gels:

The sequencing gels were prepared at 6% polyacrylamide concentration (Acrylamide and Bis-acrylamide ratio 19:1). Both the reagents used were of molecular biology grade (BDH and Sigma Chemicals, UK). Molecular biology grade urea (BDH, UK) at 7M concentration was used as a denaturant. The glass plates were thoroughly washed with light detergent and de-ionized water. The plates were dried and cleaned with ethanol. One of the plates, usually the same plate for all gels, was siliconized with Sigmacoat (Sigma Chemicals, UK). The gel thickness was kept at 0.4 mm. All sequencing gels were run on an apparatus from Bio-rad, USA. This can accommodate gels measuring 20cm X 50cm. Ammonium persulphate (400µl) and TEMED (40µl) were used as polymerization catalysts. The gels were poured using a 50ml

syringe and keeping the plates inclined at an angle of about 45°. Any bubbles were removed by gently tapping the plates. Shark-tooth combs were used for creating the sample wells.

Running the sequencing gels:

The gels were run in TBE buffer (Tris 10.8g, Borate 5.5g and Na_2EDTA 0.93g at pH 8.3). The sample wells were thoroughly washed with gel running buffer to remove any urea that constantly flows out of the gel. The labelled samples were denatured at 95°C for 2-3 minutes and kept on ice until loading. Each well was loaded with 2.5-3.0µl of reaction mixture from each tube and electrophoresis was carried out at a constant power of 50W for 2-3 hours. During the run the gel temperature remained at 50°C. At the end of the run the plates were carefully separated and the gel was fixed for 20 minutes with freshly prepared fixative containing 10% methanol and 10% acetic acid. The fixed gel was transferred to a piece of Whatman filter paper cut to a size slightly larger than the glass plates. The gel was covered with cling film and placed on a gel dryer at 80°C for 1 hour. The gel was then exposed to X-ray film (Kodak, USA) for 2-3 days.

Restriction Fragment Length Polymorphism (RFLP) analysis:

The polymorphic sites linked to the β-globin gene were analysed by first amplifying the fragment of DNA containing the site, followed by its digestion with a restriction enzyme. Table: 3.7 gives details of the polymorphic sites, the primer used for amplification and the restriction enzyme used. All of the primers were in use at the Perinatal centre, UCL, London. The general conditions for PCR were the same as ARMS except that the annealing temperatures of the primers for Aγ, 5'ψβ, 3'ψβ, Rsa-I β and Hinf-I β was 56°C and 60°C for Gγ. The number of cycles was also increased to 30. Cleavage was carried out by overnight incubation of 20µl of the amplified product at 37°C with 10 units of the restriction enzyme (Gibco BRL, UK). The digested fragments were separated on 4% NuSieve Agarose gel.

Xmn-I Polymorphism:

Xmn-I can recognize the C-T polymorphism at position -158 from the cap site of the Gγ-globin gene (Thein et al, 1988). In order to demonstrate this polymorphism, a 641bp fragment of DNA flanking the polymorphism was amplified using the following primers:

5'-GAA CTT AAG AGA TAA TGG CCT AA

5'-ATG ACC CAT GGC GTC TGG ACT AG

PCR conditions were the same as for the RFLP protocol except that the annealing temperature was increased to 60°C. The amplified fragment was digested with 10 units of Xmn-I (Boehringer, Mannheim) and the results were read after electrophoresis on 4% Nusieve Agarose gel.

Table: 3.7. Polymorphic sites linked to the β-globin gene, the restriction enzymes used for their recognition and the sequence of the primers used for amplification of the respective fragments.

Site:	**Enzyme used:**	**Primer sequence:**
Gγ-gene	Hind-III	5'-AGT GCT GCA AGA ACA ACT ACC 5'-CTC TGC ATC ATG GGC AGT GAG CTC
Aγ-gene	Hind-III	5'-GAC TAG TGC TTG AAG GGG AAC AAC 5'-CCT CTG CTG ATT CAT TTC TTA CAC
5'ψβ-gene	Hind-II	5'-TCC TAT CCA TTA CTG TTC CTT GAA 5'-ATT GTC TTA TTC TAG AGA CGA TTT
3'ψβ-gene	Hind-II	5'-GTA CTC ATA CTT TAA GTC CTA ACT 5'-TAA GCA AGA TTA TTT CTG GTC TCT
β-gene	Hinf-I	5'-TGG ATT CTG CCT AAT AAA A 5'-GGG CCT ATG ATA GGG TAA T
β-gene	Rsa-I	5'-AGA CAT AAT TTA TTA GCA TGC ATG 5'-ACA TCA AGG GTC CCA TAG AC

Gene analysis for α-thalassaemia:

Most α-thalassaemias are caused by large gene deletions. In the past these deletions were detected by laborious and expensive restriction enzyme mapping of the gene. However, the introduction of PCR has simplified the detection of gene defects in α-thalassaemia. The common α-thalassaemia-1 and 2 determinants can now be detected by PCR (Bowden et al, 1992; Dode et al, 1992; Baysal and Huisman 1994). The PCR primers are used to amplify appropriate segments of the chromosome with the deletion and the normal chromosome under identical experimental conditions. On a normal chromosome the PCR primers are far apart and therefore are unable to amplify whereas the presence of an α-thalassaemia deletion brings the

two primers sufficiently close for the amplification to take place. The amplified products can be separated by agarose gel electrophoresis.

PCR conditions for -$\alpha^{3.7}$ and -$\alpha^{4.2}$ detection:

The PCR buffer contained 67 mM Tris-HCl, pH 8.8; 16.6 mM ammonium sulphate, 0.10 mg/ml Bovine Serum Albumin, 10 mM β-Mercaptoethanol, 4.0 mM $MgCl_2$, 10% DMSO, 200 mM dNTPs, and 2.0 units of Taq polymerase (Perkin Elmer, UK). The primers and their sequences are presented in Table: 3.8. The strategy for detection is described in Fig: 3.2 (Baysal and Huisman 1994). Normally the primers A+B are far apart and can not amplify. Whereas the presence of -$\alpha^{3.7}$ deletion brings the two primers sufficiently close for amplification to take place. In the absence of the -$\alpha^{3.7}$ deletion primers A+C amplify the normal sequence. A similar approach was used for -$\alpha^{4.2}$, where primers D+E amplify the abnormal fragment if present, and D+F amplify the normal sequence.

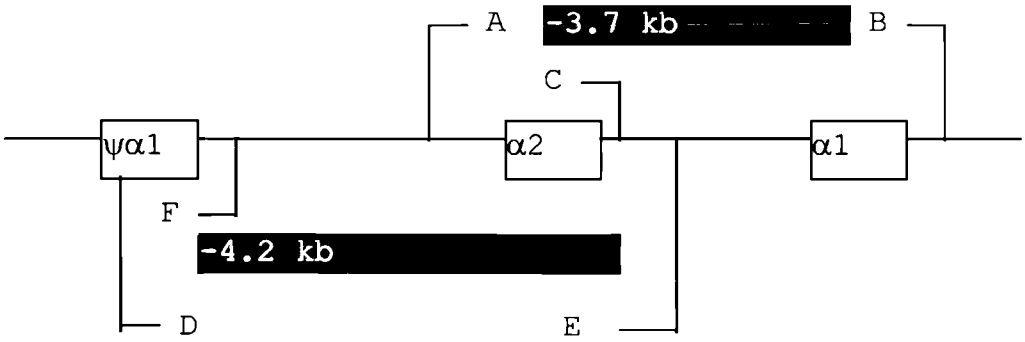


Fig: 3.2. Strategy for the amplification of segments of DNA from chromosomes with the two types of α-thalassaemia-2 deletions. The functional α2 and α1 and ψα1 genes are shown as open boxes. The solid boxes indicate the extent of the deletions. Primers A and C amplified a control fragment whereas primers A and B amplified only when 3.7kb deletion was present. Similarly, primers D and F amplified a control fragment while primers D and E amplified a fragment only when 4.2kb deletion was present (Baysal and Huisman 1994).

The PCR for detection of normal and mutant allele for -$\alpha^{3.7}$ was carried out in two different tubes because the amplified product under both conditions is of the same size, and therefore, cannot be separated on agarose gel electrophoresis. For the -$\alpha^{4.2}$ both the reactions were done in the same tube, as the amplified products considerably differ in size. The PCR was carried

out with the "Hot start" method where Taq polymerase was added when reaction mixture was above 80°C. The thermal cycling consisted of 25 cycles each of denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute and extension reaction at 72°C for 3 minutes. The extension in the last cycle was prolonged to 8 minutes. At the end of the PCR the amplified products were separated on 2% agarose gel.

Table: 3.8. Primers used for detection of α thalassaemia deletions (Baysal and Huisman 1994).

Primer ID:	**Sequence:**	**Concentration:**
A:	CTT TCC CTA CCC AGA GCC AGG TT	25 pmol/reaction
B:	CCC ATG CTG GCA CGT TTC TGA GG	25 pmol/reaction
C:	CCA TTG TTG GCA CAT TCC GGG ACA	25 pmol/reaction
D:	CCT TCC TCT CAC TTG GCC CTG AG	20 pmol/reaction
E:	CCC TGG GTG TCC AGG AGC AAG CC	15 pmol/reaction
F:	CGC CTC CCT GGA CAA GTT	15 pmol/reaction

Analysis of Short Tandem Repeats (STR):

A bulk of intergenic DNA in the human genome consists of tandemly repeating sequences whose length varies between individuals in the same population. The polymorphisms created by such elements are termed variable number of tandem repeats (VNTR) for the larger repeats and short tandem repeats (STR) for 1-6 base pair repeats (Krawczak and Schmidtke 1994). VNTRs and STRs can be analyzed by PCR (Horn et al, 1989; Urquhart et al, 1994).

STR alleles at D21S11 locus were amplified by the following pair of flanking primers (Sharma and Lit 1992):

D21S11-F 5'-GTG AGT CAA TTC CCC AAG-3'

D21S11-R 5'-GTT GTA TTA GTC AAT GTT CTC C-3'

The PCR was carried out in a 25µl reaction mixture under the same conditions as for the ARMS except that a hot start strategy was used. The thermal cycling comprised of 30 cycles each of denaturation at 93°C for 48 seconds, primer annealing at 60°C for 48 seconds and extension reaction at 72°C for 1 minute.

Allele sizing of D21S11:

In order to carry out precise allele sizing for the D21S11 locus 30 samples were run on an automated gene scanning system called ALF (Pharmacia Biotech, USA). The DNA amplified with a fluorescent labelled primer is electrophoresed on a denaturing sequencing gel. An internal lane standard is also loaded along with the test sample. The internal lane standard as well as the amplified DNA is read by the flourescent detection system comprising of a laser source. It generates a signal that is recorded as a peak and is stored in a computer.

The samples analyzed on ALF were amplified with primers in which the forward primer was labelled with flourescene. The samples were loaded on a 6% polyacrylamide gel containing 8M urea. Two internal lane standards of 100 and 300 bp (Pharmacia Biotech, UK) were also loaded with each sample. The gel was run at 1900 Volts at 42°C until the equipment had recorded the samples and the lane standards.

Once the allele sizes of several samples had been accurately determined on the ALF, suitable samples that represented most of the common alleles were chosen as controls for manual allele sizing. Each amplified sample was run on two different types of polyacrylamide gels. The samples were first run on 6% denaturing polyacrylamide gels measuring 16 X 20 cm X 1.0 mm on a Protean-II apparatus (Biorad, UK). 7µl of the amplified product and 5µl of loading dye was loaded and overnight electrophoresis was carried out at 100 volts. The gels were stained in ethidium bromide for 30 minutes and were photographed under 302 nm UV light.

The samples were also run on 6% denaturing polyacrylamide sequencing gels. The protocol for preparing the gels was the same as for sequencing, except that larger sample wells were created by using widely spaced combs. This was essential to accommodate 5µl of the sample and the loading dye. The gels were run at 500 Volts overnight.

Silver staining of polyacrylamide gels:

The glass plates were carefully separated, and the gel that usually sticks to one of the plates was fixed in 30-50 ml of freshly prepared fixing solution, containing 10% ethanol and 5% glacial acetic acid. Wrinkles in the gel, if any, were removed by gently blowing the fixative on the wrinkles with the help of a pasture pipette. The gel was then evenly covered with 0.1% silver nitrate for 20 minutes. The stain was poured off and the reaction developed by adding freshly prepared solution of 1.5% NaOH and 0.15% formaldehyde. Within 10-15 minutes the bands of DNA could be seen clearly. The gel was then washed with tap water and transferred to a clean sheet of filter paper. It was covered by cling film and left between layers of filter paper to dry.

Variable Number of Tandem Repeat (VNTR) analysis:

The VNTR sequences in the Apolipoprotein-B gene were amplified by PCR (Deka et al, 1992). The general conditions of PCR were the same as for ARMS PCR except that the annealing temperature was reduced to 55°C and the number of cycles was increased to 30. The following primers were used to amplify the VNTR sequences (Deka et al, 1992):

ApoB-F: 5'-ATG GAA ACG GAG AAA TTA TG

ApoB-R: 5'-CCT TCT CAC TTG GCA AAT AC

Electrophoresis of the amplified fragments was carried out on 2% agarose gels at 40V overnight. The gels were stained in ethidium bromide and were photographed at 302nm.

Statistical analysis:

Means, ranges, and standard deviations were calculated for the continuous variables that had normal distribution. Confidence intervals and hypothesis testing for the data that were normally distributed and for the data that were not normally distributed were calculated by parametric and non-parametric tests respectively (Altman 1991). A computer package "SPSS version 6.1.3" was used for most of the statistical analysis.

4 Epidemiology of haemoglobin disorders

Introduction:

Haemoglobin disorders are the commonest inherited disorders in Pakistan. Analysis of the situation is essential before a large scale community based prevention programme for these disorders can be initiated (Alwan and Modell 1997). The results of several studies on haemoglobin disorders in Pakistan are available (Stern et al, 1968; Hashmi and Farzana 1976; Sharma et al, 1976; Latif 1983; Saleem et al, 1985). But many of these surveys have limitations regarding the methods used, and the selection of the subjects (Khattak 1987). This chapter aims at studying the epidemiology of haemoglobin disorders in the major ethnic groups of Pakistan. The information is used to provide a basis for calculating the number of affected children born each year.

Subjects:

In this study 1345 adult males from the five major ethnic groups of Pakistan were screened for β-thalassaemia. Due to resource constraints haemoglobin electrophoresis was done only in those cases where Hb-A_2 estimation was required i.e. the cases that had MCV ≤75 fl or MCH ≤ 25pg. The subjects included, 290 (21.6%) Punjabi, 307 (22.8%) Pathan, 223 (16.6%) Sindhi, 300 (22.3%) Baluchi, and 225 (16.7%) Mohajirs. The subjects were sampled at several different places and the staff was provided by the local military hospitals. The samples from Punjabis were collected from an infantry battalion stationed at Rawalpindi. The samples from Sindhis and Baluchis were collected at the regimental centres in Hyderabad and Quetta respectively. The samples from Pathans were collected from the students of Khyber Medical College, Peshawar by a team of two volunteer doctors. The samples from Mohajirs were collected at Karachi by a mobile team of two volunteers who went from door to door for collection of samples. These samples were transported to AFIP, Rawalpindi within 12 hours of collection.

α-thalassaemia screening was carried out in one hundred randomly selected patients with β-thalassaemia major. These included 67 Punjabis and 43 Pathans. In addition, red cell indices from 264 cases of heterozygous β-thalassaemia, diagnosed by PCR, were investigated for any co-incidental α-thalassaemia. The later group of subjects comprised of the couples who requested prenatal diagnosis for thalassaemia and they all had at least one affected thalassaemic child.

Results:

β-thalassaemia:

The haematological values of 1345 adult males from the five ethnic groups are presented in Table: 4.1. The mean Hb for each ethnic group varied from 13.7 to 15.0 g/dl. Baluchis had the highest mean Hb (15.0 SD ±1.5). The number of individuals who had Hb <13.5 g/dl, varied from, 94/290 (32.4%) in Punjabis, 102/307 (33.2%) in Pathans, 98/223 (43.9%) in Sindhis, 41/300 (13.7%) in Baluchis, to 77/225 (34.2%) in Mohajirs. There were 164/1345 (12.2%) subjects who had MCV ≤75 fl or MCH ≤25 pg and required Hb-A_2 estimation (Table 4.2). Raised Hb-A_2 was found in 71/164 (43.3%) cases selected by low red cell indices. Molecular genetic analysis by multiplex PCR was done in 15 out of the remaining 96 cases because these subjects in addition to low red cell indices (MCH ≤ 25 pg or MCV ≤ 75 fl) also had Hb <9.0 g/dl. This analysis did not find any carriers.

The results of carrier screening in the ethnic groups are summarized in table 4.2. There were 71/1345 (5.3%) carriers of β-thalassaemia in all ethnic groups (95% confidence limit 4.1-6.5%). The proportion of β-thalassaemia carriers out of the individuals who had hypochromic microcytic red cell indices ranged from 39% in Punjabis, 53% in Pathans, 14% in Sindhis, 42% in Baluchis, and 52% in Mohajirs. The carrier rate in the ethnic groups was as follows: Punjabis 4.5%, Pathans, 5.2%, Sindhis 1.3%, Baluchis 9%, and Mohajirs 5.2%.

Abnormal haemoglobins:

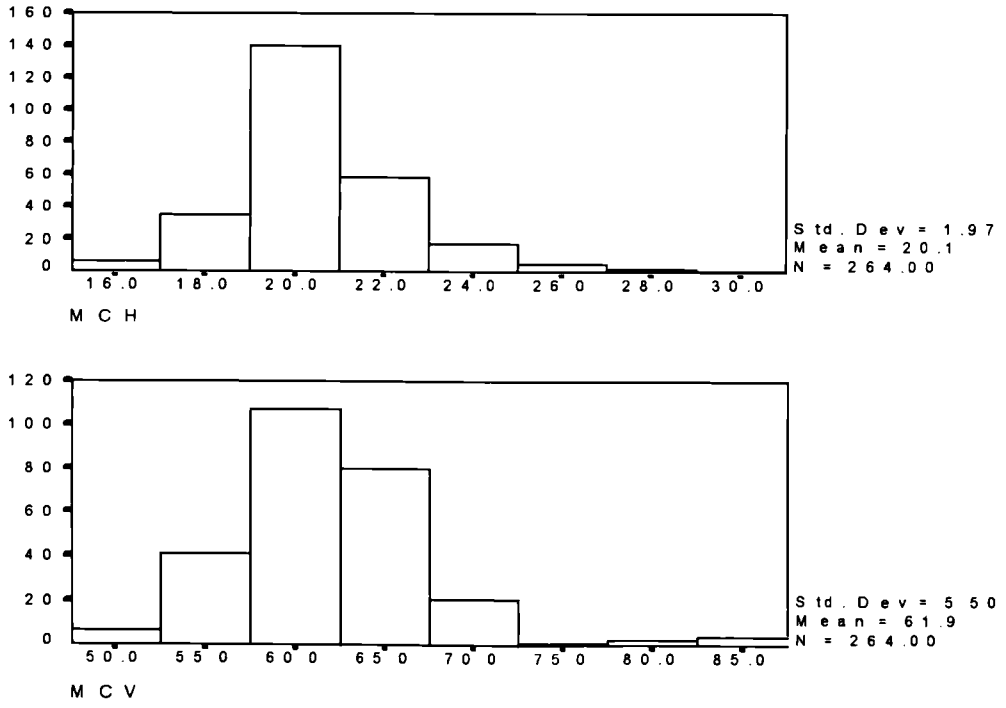
Since haemoglobin electrophoresis was done only in cases that had MCV ≤75 fl or MCH

≤25 pg, many cases of abnormal haemoglobins could not be identified. Out of the 57 Baluchi subjects, in whom electrophoresis was done, 5 (8.7%) were found to have heterozygous Hb-S. Similarly, 2/30 (6.6%) Pathans, in whom electrophoresis was done, had heterozygous Hb-S. No other abnormal haemoglobin was detected in any of the ethnic groups.

α-thalassaemia:

Heterozygous $-\alpha^{3.7}$ ($-\alpha/\alpha\alpha$) deletion was found in 5/100 (5%) cases. One case with homozygous $-\alpha^{3.7}$ ($-\alpha/-\alpha$) deletion was also seen. No case of $-\alpha^{4.2}$ deletion was observed.

The red cell indices of 264 β-thalassaemia carriers confirmed by ARMS PCR showed that 259/264 (98%) had MCH ≤ 25 pg while 256/264 (97%) had MCV ≤ 75 fl (Fig: 4.1). In the cases with MCV >75 fl or MCH > 25 pg, 5/264 (1.9%) had Cap+1 (A-C) mutation and 4/264 (1.5%) had a severe β-thalassaemia mutation. The subjects with the Cap+1 mutation had normal Hb-A_2 whereas the level in the other 4 subjects was ≥3.5%. The later four cases were suspected to have coincidental α-thalassaemia trait.



ig: 4.1 Histogram of MCV and MCH in 264 cases of β-thalassaemia trait proven by PCR.

Table: 4.1. Haematological parameters in 1345 adult males from the five major ethnic groups of Pakistan.

Ethnic group:	Hb (g/dl):		TRBC ($X10^9$/L):		MCV (fl):		MCH (pg):	
	Range:	Mean ± SD:	Range:	Mean ± SD:	Range:	Mean ± SD:	Range:	Mean ± SD:
Punjabi: (n=290)	7.0-16.8	13.9 ± 1.9	3.0-6.2	4.95 ± 0.54	54-101	85.2 ± 7.7	16-34	28.1 ± 3.3
Pathan: (n=307)	7.6-19.8	14.2 ± 1.7	4.0-6.9	5.10 ± 0.51	61-103	87.5 ± 7.1	17-35	29.1 ± 3.1
Sindhi: (n=223)	9.4-18.4	13.7 ± 1.6	3.4-7.2	4.83 ± 0.53	64-110	87.4 ± 7.6	18-39	28.6 ± 3.2
Baluchi: (n=300)	8.9-19.4	15.0 ± 1.5	3.9-7.2	5.41 ± 0.55	59-108	83.1 ± 8.0	19-36	28.1 ± 3.1
Mohajir: (n=225)	9.6-17.3	14.6 ± 1.6	3.8-7.0	4.91 ± 0.53	60-102	85.1 ± 7.2	18-33	29.2 ± 3.0
All groups: (n=1345)	7.0-19.8	14.3 ± 1.7	3.0-7.2	5.04 ± 0.53	54-110	85.7 ± 7.5	16-39	28.6 ± 3.1

Table: 4.2. Frequency of hypochromic and microcytic red cell indices and the carrier rate for β-thalassaemia in the five major ethnic groups of Pakistan.

Ethnic group:	**MCV≤75fl or MCH ≤25pg:**	**β-thalassaemia carriers:**			**95% Confidence interval:**
		Number:	**% of low MCV or MCH**	**% of total**	
Punjabi: (n=290)	33 (11.3%)	13	39.4%	4.5%	2.1-6.9%
Pathan: (n=307)	30 (9.8%)	16	53.3%	5.2%	2.7-7.7%
Sindhi: (n=223)	21 (9.4%)	3	14.3%	1.3%	0-2.8%
Baluchi: (n=300)	57 (19.0%)	27	47.4%	9.0%	5.7-12.3%
Mohajir: (n=225)	23 (10.2%)	12	52.2%	5.2%	2.3-8.1%
All ethnic groups: (n=1345)	164 (12.2%)	71	43.3%	5.3%	4.1-6.5%

Discussion:

This study is a population based survey of thalassaemia in the ethnic groups in Pakistan. Its main strengths are appropriate sample selection, representation by the major ethnic groups, and reliable methodology. However, its limitations include a relatively small sample size and the inability to screen for the abnormal haemoglobins.

Screening for thalassaemia:

β-thalassaemia trait can be suspected when MCV and/or MCH are low. Its main differential diagnosis includes iron deficiency and α-thalassaemia trait. There is a considerable overlap in the haematological picture of the three conditions. β-thalassaemia trait in most situations can be confirmed by raised Hb-A_2 and iron deficiency can be confirmed by serum ferritin (Steinberg and Adams III 1991). Identification of α-thalassaemia trait, however, requires either globin chain synthesis ratios or DNA analysis (Higgs et al, 1989). The distinction between the three conditions is important not only for treatment but also for identification and subsequent counselling of carriers. Iron deficiency is by far the commonest cause of low MCV and MCH in Pakistanis. This impression, unfortunately, has cast a shadow on the importance of low MCV and MCH caused by thalassaemia.

In this study 12% of the individuals were found to have MCV ≤75 fl and/or MCH ≤25 pg and 43% of the later population had β-thalassaemia trait. It shows that β-thalassaemia, at least in the adult healthy males, is a significant cause for low MCV and MCH. The frequency of low MCV and MCH was similar amongst all ethnic groups except in Sindhis where although 9% had this abnormality only 1.3% had β-thalassaemia trait. This may be due to concomitant α-thalassaemia in Sindhis that can mask the red cell indices in β-thalassaemia carriers (Kanavakis et al, 1982). Study of red cell indices in the British Pakistanis shows that about 11% of the adult males have MCH <23 pg, of whom about half have β-thalassaemia trait (Modell and Berdoukas 1984). MCH data from the adult male population of this study also confirm this observation (Fig: 4.2). Modell and Berdoukas (1984) have suspected that most Pakistanis who have low MCH but do not have β-thalassaemia trait may have α-thalassaemia trait. The results of low red cell indices in this study are in agreement with those of Modell and Berdoukas (1984). However, it is questionable whether all individuals with low MCV or MCH who are

not β-thalassaemia carriers have α-thalassaemia because many of them might also be iron deficient. Screening for the common α-thalassaemia determinants in 100 individuals investigated in this study showed that 6% carried $-\alpha^{3.7}$ deletion (-α/αα:5 and -α/-α:1). The study of α-thalassaemia although very small indicates that the majority of the individuals who have low MCV or MCH and are not β-thalassaemia carriers may have iron deficiency. Population-based data on α-thalassaemia in Pakistan are very scanty. The only two studies by Khan and Hayee (1986) and Zahur-ur-Rehman et al, (1991) show carrier rates of 0.94% and 2.4% respectively. These studies also support that α-thalassaemia is not a significant factor in causing low MCV or MCH in the Pakistanis.

The carrier rate for deletional α-thalassaemia-2 was found to be 6%. At this rate one would expect 0.4% of the population to be homozygous for α-thalassaemia-2. However, red cell indices in 264 β-thalassaemia carriers (confirmed by PCR) showed that 97% had MCV≤75fl and 98% had MCH ≤25pg. In the 3% with red cell indices in the normal range half had Cap+1 mutation. This suggests that interference due to co-inheritance of α-thalassaemia (-α/-α) may be present in only 1.5% of the cases. If the deletional forms of α-thalassaemia is seen in 0.4% then the remaining 1% must be non-deletional in type. The combined incidence of deletional as well as non-deletional forms of α-thalassaemia in Pakistan may be around 12%, a rate at which about 1.5% of the population may be homozygous for α-thalassaemia trait (deletional or non-deletional).

In populations where α-thalassaemia is common it can cause significant problems in identification of β-thalassaemia carriers (Kanavakis et al, 1982). Another diagnostic problem may be caused by silent mutations (Cao et al, 1994). The results of this study indicate that both α-thalassaemia and silent mutations may not be very significant problem in Pakistani population. However, in working out a strategy for detection of at risk couples it may be useful to investigate by DNA methods all those individuals whose partners are known carriers of β-thalassaemia.

Modell and Berdoukas (1984) suggested that for all practical purposes the finding of MCH ≥25 pg should exclude β-thalassaemia trait in Pakistanis. The same cut off limit for MCH was

used in this study for identifying individuals requiring Hb-A_2 estimation. This approach, although cost effective, might have missed about 3% of β-thalassaemia carriers who either had concomitant α-thalassaemia or silent mutations. However, in an epidemiological survey this would not make a significant difference.

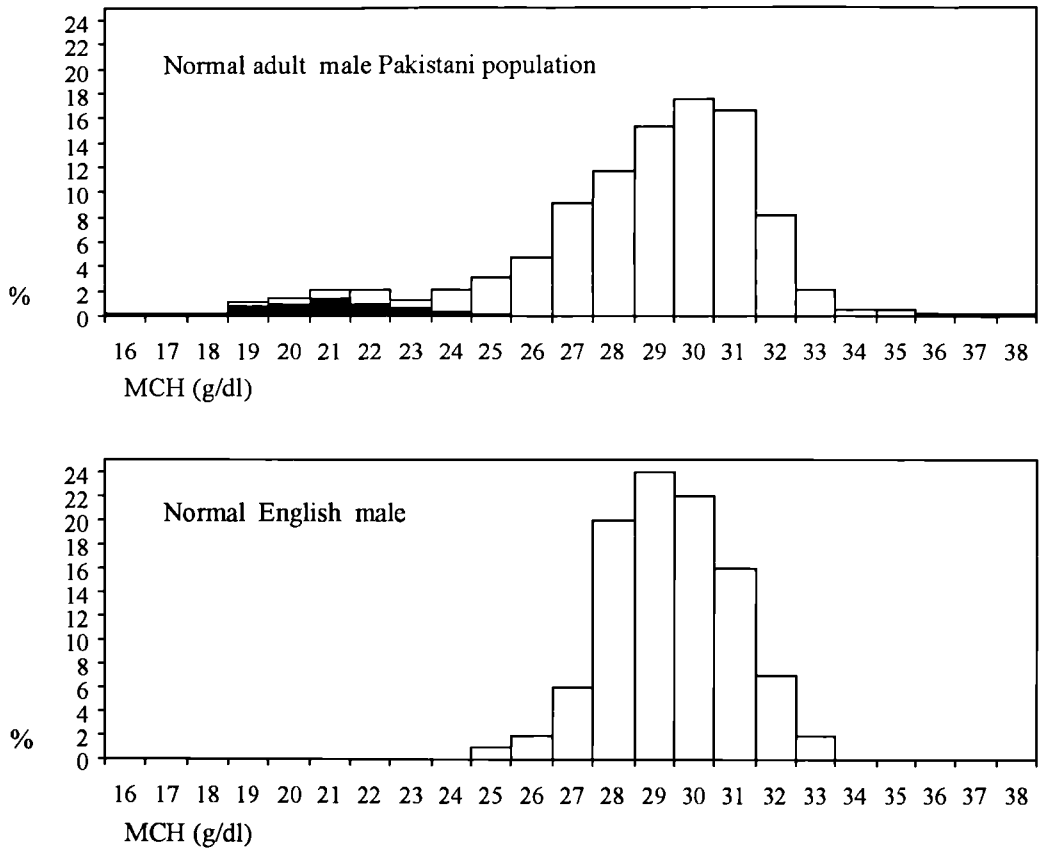


Fig: 4.2. A comparison of MCH values from the normal adult male Pakistanis (this study) and a similar English population (Modell and berdoukas 1984). The Pakistanis have a long tail in the low MCH region. The solid area in the histogram represents the proportion of β-thalassaemia carriers.

β-thalassaemia carrier rates in the ethnic groups:

The study by Khattak and Saleem (1992a) gave an indication that the β-thalassaemia carrier rate is different in Punjabis (3.3%) and Pathans (8.0%) (Table: 4.1). Apart from the later study no information is available on the carrier rate in any of the ethnic groups in Pakistan. It is important to investigate the carrier rate in each group because β-thalassaemia genes may be unevenly distributed amongst the ethnic groups. Moreover, the frequency of consanguineous marriage is also different between the ethnic groups (Bittles 1994; Wahab and Ahmad 1996).

This could affect the calculation of annual birth rate of thalassaemia major in each group. The overall β-thalassaemia carrier rate of 5.3% (95% confidence limits: 4.1-6.5%) found in this study is in agreement with the generally accepted figures (WHO 1985). The carrier rate in the ethnic groups varied considerably. The rate was lowest in Sindhis (1.3%) and highest in Baluchis (9%). The carrier rate in Sindhis may be spuriously low due to coincident α-thalassaemia. This issue, however, remains open for further investigations. Baluchis are a well-defined group who are mostly descendants of a few tribes that originally migrated from Iran to their present location in 700 B.C. (Bokhari 1975). It is likely that the high gene frequency for thalassaemia in Baluchis is due to a founder effect (Bodmer and Cavalli-Sforza 1976).

Abnormal haemoglobins:

A major limitation of this study is the inability to screen for abnormal haemoglobins. However, other published reports indicate that abnormal haemoglobins are not a significant problem as compared to β-thalassaemia. Hashmi and Farzana (1976) found the following carrier rates in 1224 individuals from Karachi: Hb-D 0.65%, Hb-E 0.16%, and Hb-S 0.08%. The largest study of 5000 Pakistani Armed Forces personnel by Sharma et al, (1976) showed that 39 (0.78%) had an abnormal haemoglobin including Hb-D (0.42%), Hb-E (0.18%), and Hb-S (0.18%). A study of 500 individuals from the northern parts of Pakistan showed that 1.2% carry heterozygous Hb-D, and 0.2% were carriers of Hb-E (Khattak and Saleem 1992b).

Calculation of the number of annual births of thalassaemia major:

Carrier frequency of thalassaemia can be used to calculate the annual number of births of affected children. This information is essential prerequisite in planning a national programme for control and prevention of the disorder (Alwan and Modell 1997).

Theoretical considerations:

The annual birth rate for thalassaemia major can be calculated by the Hardy Weinberg analysis of the carrier rate (Bodmer and Cavalli-Sforza 1976). According to the Hardy Weinberg law, the number of heterozygotes and the homozygotes for a trait in a randomly mating population is: $p^2 + q^2 + 2pq = 1$. Where p and q are the frequencies of the two alleles under study. If p and q are the frequencies for the normal and β-thalassaemia genes in a population then p^2, q^2 and

$2pq$ will represent the proportion of normals, homozygotes and heterozygotes respectively. In a population where mating is not random, the proportion of heterozygotes is reduced in comparison to Hardy-Weinberg proportions by an amount $2Fpq$, while that of homozygotes is increased by Fpq where F is the inbreeding coefficient. The actual number of homozygotes will be equal to $q^2 + Fpq$ (Bodmer and Cavalli-Sforza 1976).

Taking the carrier rate for β-thalassaemia as 5% (0.05) its gene frequency (p) will be 0.025 (1/2 of the carrier rate because each carrier has one normal and one β-thalassaemia gene) and the frequency of normal gene (q) will be $1-p$ (0.975). The number of β-thalassaemia homozygotes (p^2) per thousand will be 0.625. This figure would require a correction for the frequency of consanguineous marriages. The average inbreeding coefficient (F) of the Pakistani population is 0.028 (Bittles 1994). The actual number of β-thalassaemia homozygotes will be equal to $p^2 + Fpq$ i.e. 0.625 + 0.683 = 1.308 per thousand.

Calculations based on the already published work:

Several studies have focused attention on carrier rate of β-thalassaemia in Pakistan. Table 2.2 gives a summary of the studies so far carried out on Pakistanis. Stern et al, (1968) were the first to report a 4% carrier rate for β-thalassaemia amongst 129 Pathans. The studies conducted thereafter have reported carrier rates ranging from 1.4-9.6%. Variability in the results is mostly due to lack of adequate laboratory facilities, technical difficulties in carrier detection, and inappropriate selection of the target population (Khattak 1987). WHO (1985) have reported an overall β-thalassaemia carrier rate of 5%.

The estimated number of annual thalassaemia major births in Pakistan, based on 5% carrier rate in 137 million population with annual birth rate of 2.8% (Economic survey 1995-1996) and a coefficient of inbreeding of 0.028 (Bittles 1994) will be 5014. This estimate is based on the assumption that the carrier rate and the frequency of consanguineous marriage in all ethnic groups is similar.

Revised calculations based on the results of this study:

This study shows that the carrier rates amongst the ethnic groups are different. The coefficient of inbreeding also varies considerably between the groups (Table: 2.1). It is therefore expected that estimates of the annual birth rate for thalassaemia major would also be different in each ethnic group. Table: 4.3 gives a detailed picture of the calculation of number of annual births of thalassaemia major in each ethnic group. The population of Pakistan is estimated at 137 million and the annual birth rate is 2.8% (Economic Survey 1995-1996). The last census was carried out in 1981. The latest information on the population of ethnic groups is not available. For the purpose of calculation the population of each group has been taken in the same proportion as these were found in the 1981 census.

The revised calculations show 4550 annual new births of thalassaemia major (1.2/1000) in Pakistan (Table 4.3). The maximum number of births is expected in Punjabis (1.124/1000) which also is the largest ethnic group (80 million). The highest birth rate is expected in Baluchis (4.3/1000) due to a high carrier rate and high frequency of consanguineous marriages. The birth rate amongst Sindhis (0.324/1000) is low because of a very low carrier frequency (1.3%) found in this group. As discussed earlier the carrier rate in Sindhis might be spuriously low because of possible coincidental α-thalassaemia. Unpublished data from the Fatimid thalassaemia centre in Karachi, that looks after the majority of Sindhi patients, suggests that the number of Sindhi patients with thalassaemia are not less than the patients from other ethnic groups.

Consanguineous marriage and thalassaemia:

Consanguineous marriage increase the frequency of recessive disorders. The increase, however, is most marked in disorders that are rare (Modell and Kuliev1992). Table: 4.3 gives an estimate of the number of thalassaemia births with and without correction for the consanguineous marriage. It is clear that the number of births in the absence of consanguineous marriage is lower (2442) than in their presence (4550). It is apparent that even in the complete absence of consanguineous marriage there would still be approximately 2500 annual births of thalassaemia major.

Table: 4.3. Calculation of the expected number of births of children with β-thalassaemia major per year in the five ethnic groups.

Ethnic group:	**Population: (millions)**	**β-thalassaemia Carrier rate:**	**β-thalassaemia gene frequency (p)**	**Normal gene frequency (q)**	**Coefficient of inbreeding (F)[$]**	**Number of children with thalassaemia major per 1000 new births**		**Annual Birth rate (%)**	**Total new births per year (millions)**	**Number of children with thalassaemia major born per year with reference to the practice of customary consanguineous marriages**	
						p^2	$(p^2 + Fpq)$			Absent	Present
Punjabi	80	4.5%	0.0225	0.977	0.028	0.506	1.124	2.8%	2.184	1365	2454
Pathan	20	5.2%	0.026	0.974	0.0164	0.676	1.091	2.8%	0.56	378	610
Sindhi	15	1.3%	0.0065	0.994	0.0437	0.042	0.324	2.8%	0.42	18	136[£]
Baluchi	7	9.0%	0.045	0.955	0.0532	2.025	4.311	2.8%	0.196	397	844
Mohajir	15	5.2%	0.026	0.974	0.0209	0.676	1.205	2.8%	0.42	284	506
Total	137	-	-	-	-	-	-	-	-	2442	4550

[$] F values are based on Table: 2.1.
[£] See page 78 para 2 for comments.

Calculation of the number of patients with abnormal haemoglobins:

Since abnormal haemoglobins were not studied, their calculations are based on the already published data. The largest study of abnormal haemoglobins in Pakistanis is that of Sharma et al, (1976) which reported an incidence of 0.18% each for Hb-S and Hb-E. According to these figures it is estimated that only three new homozygotes each of Hb-S and Hb-E will be born every year if there was no consanguineous marriage. However, the expected number of homozygotes in the presence of consanguineous marriage is estimated at 99 each for Hb-S and Hb-E. The number of new births of compound heterozygotes of abnormal haemoglobins and/or thalassaemia per year is estimated at 368 (Hb-S/β-thalassaemia: 181, Hb-E/β-thalassaemia: 181, and Hb-E/Hb-S: 6). The correction for consanguineous marriages on the birth rate of compound heterozygotes is more difficult to apply. In fact, it is expected that consanguineous marriage would reduce their incidence. The total number of new cases of a clinically significant abnormal haemoglobins, including compound heterozygotes with thalassaemia, is expected to be 560.

The effect of consanguineous marriage on the birth rate of Hb-S and Hb-E is marked because both are rare in Pakistan. Only three homozygous SS or EE births per year are expected without any correction for consanguineous marriage as compared to 99 per year when the correction is applied. This highlights the importance of consanguineous marriages in affecting the birth rate of rare recessive disorders.

Estimated total number of patients and pregnancies at risk:

The number of births of a clinically significant haemoglobin disorder per year in Pakistan is estimated at 5110 (thalassaemia: 4550 and abnormal haemoglobins 560). This corresponds to an annual birth rate of 1.35/1000 new births. Thalassaemia major represents the bulk of the problem (Fig: 4.3). The estimated number of pregnancies at risk would be around 20440.

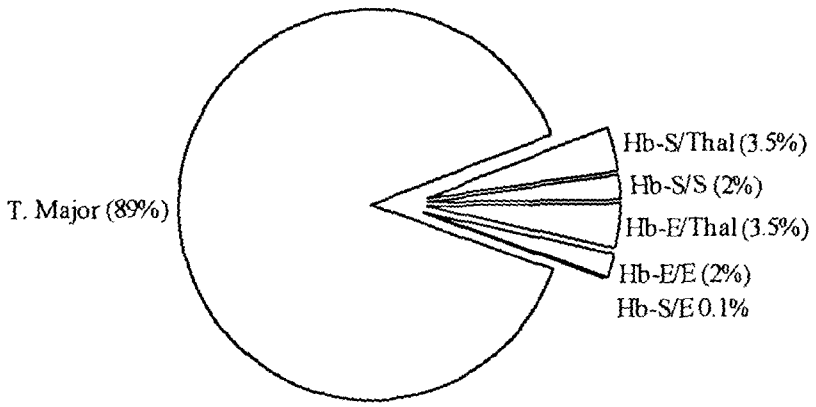


Fig: 4.3. The estimated annual births of a clinically significant haemoglobin disorder in Pakistan.

5 Screening for haemoglobin disorders

Introduction:

Initiating a community based carrier screening programme for β-thalassaemia in Pakistan is handicapped by resource constraints, low rate of literacy and lack of organization of the health care system. In order to evaluate the feasibility of a carrier screening strategy, a pilot study of two approaches was carried out i.e. screening in pregnancy and testing of extended family members when a patient with thalassaemia major was identified. This chapter describes the results, advantages and disadvantages of the two approaches.

Subjects:

Screening in pregnancy:

A total of 350 pregnant women, who reported at the antenatal clinic of Holy Family Hospital, Rawalpindi, were included in the study. The husbands of the women found to be carriers of β-thalassaemia were also screened at a subsequent stage.

Targeted screening:

A total of 16 families with a haemoglobin disorder were offered carrier screening. The screening service was offered at the homes of family members. The selection criteria, other than voluntary participation, were the ease of availability of the maximum number of family members.

Eight families without an apparent history of a haemoglobin disorder were also offered screening for inclusion as controls. The only selection criterion was the ease of availability of the maximum number of family members. The control families belonged to the members of the laboratory staff working at AFIP who were aware of the implications of screening for haemoglobin disorders.

Methods:

Sampling procedures:

Antenatal screening:

A volunteer lady doctor did the screening in the antenatal clinic at Holy Family Hospital, Rawalpindi. At the time of screening a note was made about the ethnic group, consanguinity (husband and wife) and the number of weeks of gestation.

Screening in the families:

The samples for screening in the families were collected by a team comprising of two other assistants and myself. The two assistants were male laboratory technicians whose basic education was FA (equivalent to A levels). They were briefed about drawing pedigrees. They also agreed to collect samples from their own families as controls.

A box containing about 100 disposable plastic syringes (5 ml), approximately 100 blood collection tubes, spirit swabs, tourniquet, sample racks for holding about 100 tubes, a sketch book and pencil for drawing the pedigree were always kept ready for a field trip. At each visit the presence of at least two members of the team was considered essential. After selecting a family for sampling a meeting with one of the family member was arranged. In the subject families the key person was either the father, mother or a grandparent of the affected child. In the control families the person who was first offered screening acted as the resource person. The importance of carrier screening was fully explained to the key person(s) and a sketch of the pedigree was drawn. Later the sketch was transferred to a computer with the help of a pedigree drawing software "Cyrillic Version 2.0". A suitable time and date for sampling was arranged by mutual agreement with the family members.

Genetic counselling:

Genetic counselling was carried out according to the internationally accepted guidelines (Harper 1993). It was non-directive, and was aimed at providing information to the individual or the couple so that they could make an informed choice. Particular care was taken to maintain confidentiality.

The results of antenatal screening were communicated to the women and the importance of screening their husbands was explained. The results of screening in the families were given either to the carriers themselves or to the parents of very young subjects. Counselling of the very young individuals was done through the parents. The significance of being a carrier and the possibility of planning marriages in the future were verbally explained. An easy to understand booklet in Urdu was provided to those who could read.

Results:

Screening in pregnancy:

A total of 350 pregnant women were screened. This included 284 (81%) Punjabi, 40 (11.5%) Mohajir and 26 (7.5%) Pathan women. Fig: 5.1 shows the number of weeks of gestation of the women at the time of screening. Only 18% of the women were in the 1st trimester.

A summary of the haematological parameters of the women screened is presented in Table: 5.1. The frequency distribution of MCV and MCH shown in Figs: 5.2 and 5.3. There were 66/350 (19%) women who had MCV ≤75 fl or MCH ≤25 pg and required Hb-A_2 estimation. Hb-A_2 above normal (≥3.5%) was found in 15 women. In 12 out of the remaining 51 women who either had Hb-A_2 in the borderline rage (3.0-3.4%) or their Hb was <9.0 g/dl DNA analysis by multiplex ARMS was carried out to establish the diagnosis. As a result of DNA analysis two more women were diagnosed as β-thalassaemia trait. One had Hb 9.9 g/dl, MCV 63.5 fl and MCH 21.7 pg and the other had Hb 9.7 g/dl, MCV 58.1 fl MCH 19 pg. There were 17/350 (4.9%) β-thalassaemia carriers and only 2/17 carriers were in the first trimester of pregnancy. The remaining 49 women were not investigated further. It is expected that 12% of them would have α-thalassaemia and the remaining would have iron deficiency.

Both carrier or non-carrier women with low MCV and/or MCH had no difference in the level of Hb (Table: 5.1). However, microcytosis (low MCV) and hypochromia (low MCH) was more marked in the carrier women than in the non-carriers. Similarly, TRBC values were also higher in the carrier women than the non-carriers.

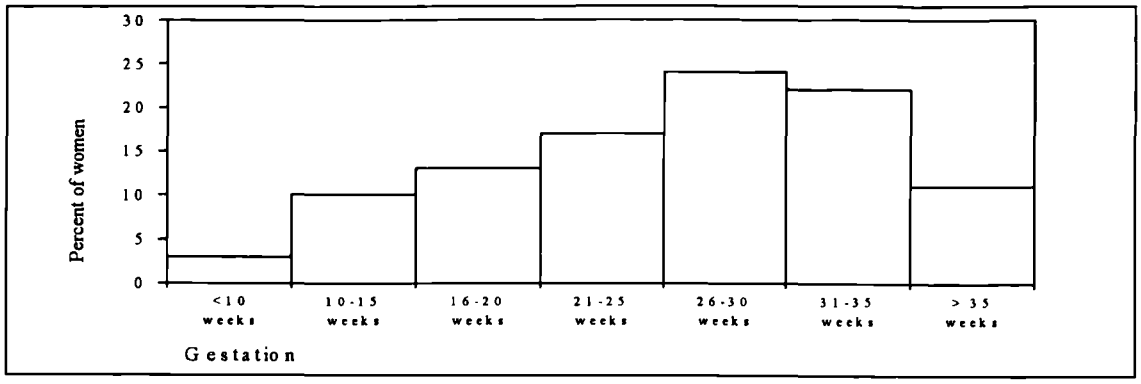


Fig: 5.1. Number of weeks of gestation at the time of screening in 350 women.

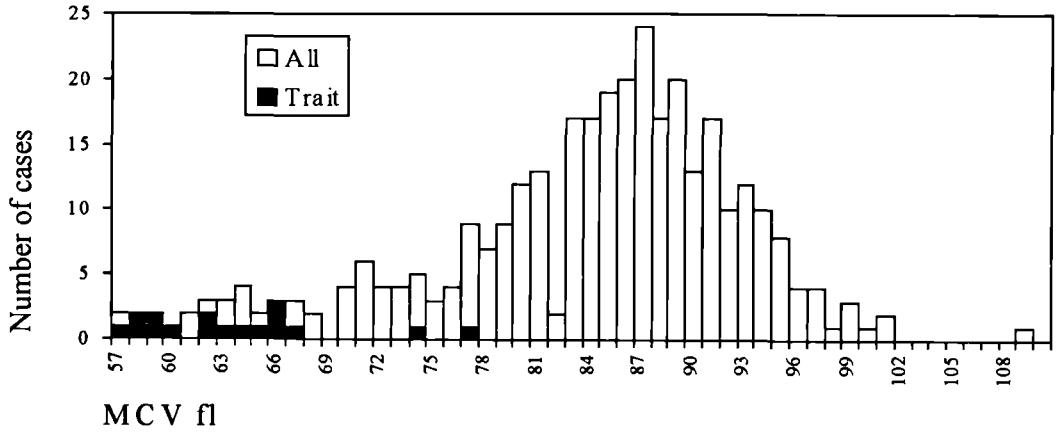


Fig: 5.2. Frequency distribution of MCV in 350 pregnant women screened for thalassaemia in an antenatal clinic.

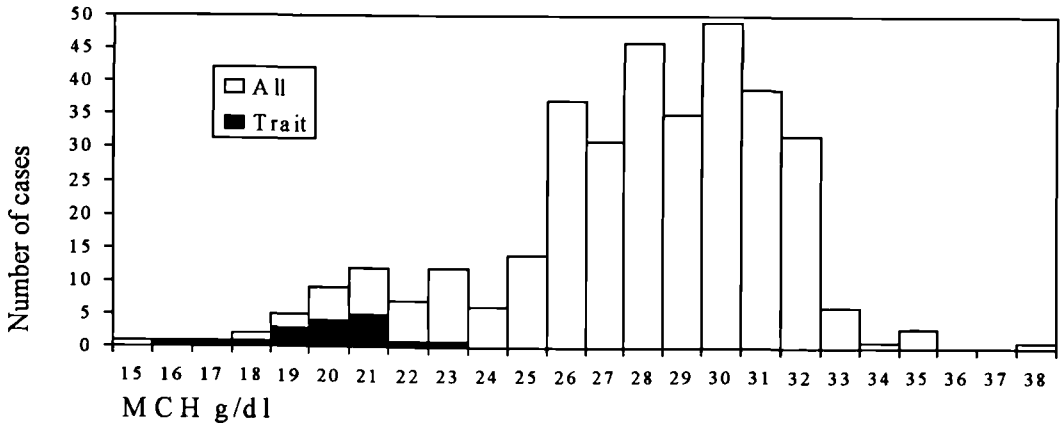


Fig: 5.3. Frequency distribution of MCH in 350 pregnant women screened for thalassaemia in an antenatal clinic.

Table: 5.1. Haematological parameters in 350 pregnant women in whom screening for thalassaemia was done in an antenatal clinic.

Pregnant women (n):	**Hb (g/dl):**		**TRBC ($X10^9$/L):**		**MCV (fl):**		**MCH (pg):**	
	Range:	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**
All (350):	5.0-15.2	11.2 ± 1.5 (11.1-11.4)	2.80-5.91	4.09 ± 0.46 (4.04-4.13)	56-109	83.7 ± 8.8 (82.8-84.6)	15.3-37.8	27.8 ± 3.7 (27.4-28.1)
Normal (not trait) (333):	5.6-15.2	11.3 ± 1.5 (11.2-11.5)	2.80-5.41	4.05 ± 0.41 (4.04-4.09)	59-109	84.7 ± 7.8 (83.8-85.5)	15.3-37.8	28.1 ± 3.3 (27.8-28.5)
MCV ≤75fl or MCH ≤25 pg (66):	5.0-11.7	9.2 ± 1.4 (8.8-9.5)	2.80-5.91	4.29 ± 0.60 (4.16-4.46)	56-92	69 ± 6.9 (67.3-70.8)	15.3-26.3	21.4 ± 2.4 (20.7-21.8)
MCV ≤75fl or MCH ≤25 pg and nothal trait (49):	5.6-11.4	9.0 ± 1.3 (8.6-9.4)	2.84-5.18	4.13 ± 0.46 (4.00-4.26)	59.3-92	71.1 ± 6.36 (69.3-73.0)	15.3-24.9	21.8 ± 2.4 (21.1-22.4)
β-thalassaemia trait (17):	5.0-11.7	9.6 ± 1.5 (8.8-10.4)	3.02-5.91	4.81 ± 0.7 (4.45-5.17)	56-77	63 ± 5.6 (60.6-66.3)	16.4-22.7	19.9 ± 1.6 19.1-20.7)

SD: Standard deviation; CI: Confidence Interval.

The husbands of 17 β-thalassaemia carrier women were also screened and none was found to be a carrier. The husbands of 5/17 (29%) were first cousins, 5/17 (29%) were more distantly related while 7/17 (42%) were unrelated.

Targeted carrier screening:

Families with a known history of haemoglobin disorder (subjects):

Six out of the 16 selected families with a history of a haemoglobin disorder refused testing for the following reasons:

1. It will be very difficult to get every one together.
2. Other people say that we are all right why should we get tested?.
3. I will decide after consulting the elder brother.
4. Other relatives do not understand this and it will be difficult to get them together.
5. We will not allow you to come to our village.
6. It is very difficult to ask every one to give blood for testing.
7. We may have problems in arranging marriages of our children especially daughters.
8. We would like to be tested but some of our relatives think that it is not good because other people might think this family has some problem for which a "team of doctors" is coming from Rawalpindi to test them.

Families without history of haemoglobin disorder (controls):

Out of the eight individuals who were offered screening of their families, five agreed. The remaining three found it difficult to collect samples from the other family members. All three felt it was difficult to ask every one else in the family to give blood for no obvious reason.

Description of the families:

Subject families:

Table 5.2 summarizes the family condition, location, ethnic group, Biradri/Tribe, number of family members, and the number of individuals tested in the 10 subject families. Seven

families were targeted because at least one child with thalassaemia major was present, one family was targeted because of thalassaemia trait in a family member, and two families had children with sickle cell disease. Eight subject families were Punjabis and two were Pathans. Three families were from urban localities while seven were from rural areas. There were 846 alive members in the 10 families. On an average there were 85 living members per family. Out of 846 members 591 (70%) were tested.

Control families:

The five control families had no previous history of a haemoglobin disorder in the family (Table: 5.3). These included two Punjabi and three Pathan families. All five were from the rural areas. In the five control families there were 609 living individuals and the average number of individuals per family was 122. In the five families 397/609 (65%) individuals were screened. On an average there were 97 individuals per family in the 10 subject and 5 control families.

Pedigrees:

Pedigrees of the subject and the control families are shown in Fig: 9.4 to 9.17 (Chapter 9).

Time taken in sampling:

The sampling time of a complete family varied from one day to one month depending on the size and location of the family, distance from AFIP (place where testing was done), and the distribution of the family members. The two families (No: 1 and 2), resident in the urban areas, were particularly difficult to sample. It took 4-5 visits each to collect their samples. The main cause of delay was the adult males and school going children who were often busy in their daily routine. The best time to sample such families was late evening hours when most members were available. The sampling was easiest in the rural areas where it usually took a few hours to sample the whole family. The people in small villages were more co-operative and welcoming than in the urban areas.

In all families the role of one key member, usually a parent or a grandparent of the affected child, was found to be very important. The sampling was greatly facilitated when an assertive or influential person was available. In fact, some of the families who refused

screening, did so due to the lack of a key member who could convince his relatives to have screening.

Haematological parameters:

Haematological parameters were evaluated in 480 individuals from the eight subject families and 397 individuals from the five control families. The samples from 111 individuals of the two subject families with sickle cell disorders (No: 9 & 10) haemolysed due to extreme hot weather and lack of refrigeration facilities in the area of collection. These samples therefore could not have red cell indices done. However, it was possible to prepare haemolysates for electrophoresis on their samples.

Table: 5.4 presents a comparison of the haematological parameters (Hb, TRBC, MCV, and MCH) between the individuals of the subject and the control families. The Hb level of adults as well as children from the subject and the control families were not different. TRBC of adult males and children from the subject families was significantly higher than the controls (no overlap in 95% CI). The difference for the adult females was not significant. Similarly, MCV and MCH in the adults as well as the children from the subject families were significantly lower than the controls. Fig: 5.4 & 5.5 show a comparison between the frequency distribution of MCH in adult males, adult females and children $\leq$15 years from the subject and the control families.

Table: 5.5 presents a summary of the haematological parameters in the individuals from the subjects and the control families grouped according to age, sex and β-thalassaemia carrier status. Hb levels in all groups were lower in β-thalassaemia carriers than the non-carriers. Similarly, TRBC was higher and MCV and MCH were lower in β-thalassaemia carriers than the non-carriers. Amongst the β-thalassaemia carriers the adult females as well as the children had significantly lower Hb as compared to the adult males. The children with β-thalassaemia trait had lower MCV and MCH than the adult carriers.

Table: 5.2. The family condition, location, ethnic group, Biradri/Tribe, and the number of family members in the 10 subject families with a known history of haemoglobin disorder.

Family ID:	Family Condition:	Place:	Location:	Ethnic group:	Biradri/ Tribe:	Total alive family members:	Number tested:
No: 1	β-thalassaemia	Rawalpindi Lahore & Faisalabad	Urban	Punjabi	Sheikh	199	138
No: 2	β-thalassaemia	Rawalpindi	Urban	Punjabi	Awan	85	85
No: 3	β-thalassaemia	Sagra (Taxilla)	Rural	Punjabi	Khattar	55	51
No: 4	β-thalassaemia	Kotha Kalan Siahala (Rawalpindi)	Rural	Punjabi	Rajpoot	48	41
No: 5	β-thalassaemia	Talagang	Rural	Punjabi	Awan	69	45
No: 6	β-thalassaemia	Chakwal	Urban	Punjabi	Awan	60	42
No: 7	β-thalassaemia	Utch (Bahawalpur)	Rural	Punjabi	Khawaja	80	20
No: 8	β-thalassaemia	Malakwal (Chakwal) Lahore	Rural/Urban	Punjabi	Sipra	98	58
No: 9	Sickle	Kaka Khail (Tank)	Rural	Pathan	Bhittani	79	48
No: 10	Sickle	Kari Wadatti (Tank)	Rural	Pathan	Bhittani	73	63
Total:	-	-	-	-	-	846	591

Table: 5.3. The family condition, location, ethnic group, Biradri/Tribe, and the number of family members in the five control families without a known history of haemoglobin disorder.

Family ID:	**Condition:**	**Place:**	**Location:**	**Ethnic group:**	**Biradri/ Tribe:**	**Total alive family members:**	**Number tested:**
No: 11	Normal	Khamidan Banda (Karak)	Rural	Pathan	Khattak	120	48
No: 12	Normal	Danouri (Sialkot)	Rural	Punjabi	Gujar	72	57
No: 13	Normal	Mulla Zai (Tank)	Rural	Pathan	Marwat	117	111
No: 14	Normal	Kotki Berouni (Mianwali)	Rural	Pathan	Khattak	148	131
No: 15	Normal	Bhojanwala (Sahiwal)	Rural	Punjabi	Noon	152	50
Total:	Normal	-	-	-	-	609	397

Table: 5.4. A comparison between the haematological parameters of individuals from the subject and the control families.

Group of individuals:	Hb (g/dl):		TRBC ($X10^9$/L):		MCV (fl):		MCH (pg):	
	Range:	Mean ± SD (95% CI):	Range:	Mean ± SD (95% CI):	Range:	Mean ± SD (95% CI):	Range:	Mean ± SD (95% CI):
Adult male:								
Subjects (n=139):	8.7-16.8	13.6 ± 1.7 (13.4-13.9)	3.17-7.73	5.34 ± 0.80 (5.30-5.57)	56-100	77.8 ± 12.3 (75.7-79.8)	17.5-34.3	25.6 ± 4.92 (24.7-26.4)
Controls (n=74):	7.0-16.7	13.5 ± 2.12 (13.0-14.0)	3.00-6.20	4.93 ± 0.64 (4.79-5.08)	54-101	84.3 ± 8.7 (82.3-86.3)	16.0-31.3	27.3 ± 3.16 (26.6-28.0)
Adult females:								
Subjects (n=152):	7.5-15.0	11.6 ± 1.68 (11.3-11.8)	3.31-6.67	4.81 ± 0.66 (4.7-4.92)	49-100	76.0 ± 12.5 (74.0-78.0)	13.7-33.4	24.4 ± 4.65 (23.6-25.1)
Controls (n=112):	5.3-16.8	11.3 ± 1.77 (11.0-11.7)	2.90-6.10	4.39 ± 0.50 (4.30-4.83)	55-109	82.7 ± 10.6 (80.8-84.7)	14.0-34.8	26.0 ± 3.72 (25.3-26.7)
Children ≤ 15 years:								
Subjects (n=189):	3.5-15.6	11.2 ± 1.83 (10.9-11.4)	2.34-7.22	4.82 ± 0.77 (4.70-4.93)	48-100	73.0 ± 11.7 (71.4-74.7)	10.8-33.9	23.7 ± 4.94 (23.0-24.4)
Controls (n=211):	5.0-15.6	11.3 ± 1.83 (11.0-11.5)	3.10-5.90	4.52 ± 0.43 (4.46-4.58)	46-103	79.4 ± 10.9 (77.9-8.9)	11.0-33.5	25.1 ± 4.01 (24.6-25.7)

SD: Standard Deviation; CI: Confidence Interval

Table: 5.5. Haematological parameters in 877 individuals from the subject and the control families.

Group of individuals:	**Hb (g/dl):**		**TRBC ($X10^9$/L):**		**MCV (fl):**		**MCH (pg):**	
	Range:	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**
Normal adult males: (n=164)	7.0-16.8	13.9 ± 1.9 (13.6-14.2)	3.00-6.20	4.96 ± 0.55 (4.87-5.04)	54-101	85.3 ± 7.7 (84.1-86.5)	16.0-34.3	28.1 ± 3.3 (27.6-28.6)
Adult males with β-thal trait: (n=52)	8.7-15.0	12.6 ± 1.2 (12.3-12.9)	4.78-7.73	6.23 ± 0.62 (6.06-6.40)	56-70	63.4 ± 3.4 (62.5-64.4)	17.5-22.9	20.2 ± 1.3 (19.8-20.5)
Normal adult females: (n=216)	5.3-16.8	11.7 ± 1.8 (11.5-11.9)	2.90-6.10	4.45 ± 0.48 (4.39-4.52)	55-109	82.9 ± 9.6 (81.6-84.2)	14.0-34.8	26.4 ± 3.6 (25.9-26.9)
Adult females with β-thal trait: (n=52)	8.1-12.2	10.5 ± 1.1 (10.2-10.8)	3.61-6.67	5.39 ± 0.62 5.22-5.67)	49-74	62.1 ± 4.8 (60.8-63.5)	13.7-24.5	19.6 ± 2.0 (19.0-20.1)
Normal children ≤15 yrs: (n=342)	5.0-15.6	11.5 ± 1.8 (11.3-11.7)	3.10-6.10	4.55 ± 0.45 (4.50-4.60)	46-103	79.0 ± 10.1 (77.9-80.1)	11.0-33.9	25.3 ± 4.0 (24.9-25.8)
Children ≤15 yrs with β-thal trait (n=51)	3.5-11.9	10.0 ± 1.4 (9.5-10.4)	3.23-7.22	5.59 ± 0.74 (5.38-5.80)	48-74	58.6 ± 5.9 (57.0-60.3)	10.8-23.6	18.0 ± 2.6 (17.3-18.7)

SD: Standard Deviation; CI: Confidence Interval

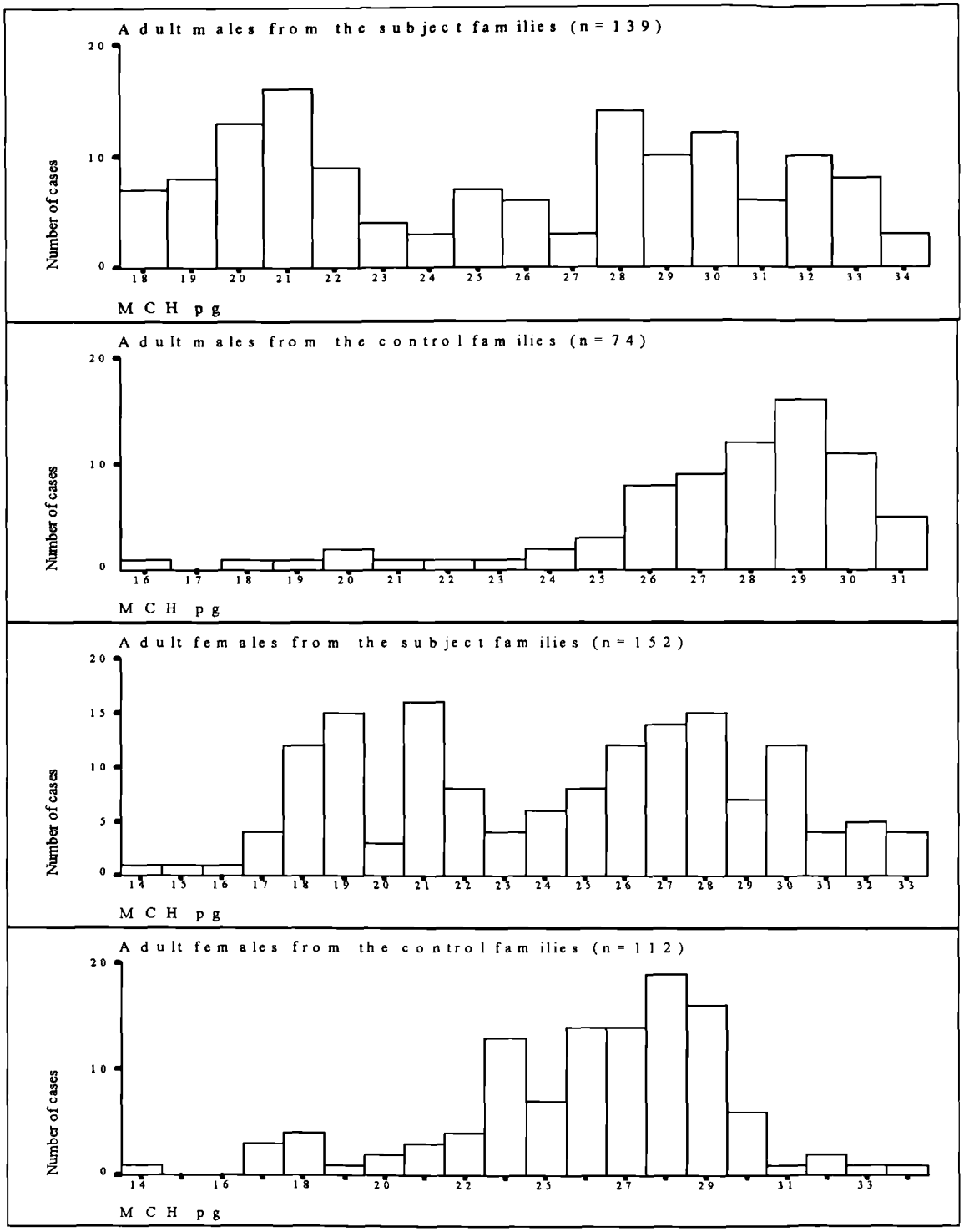


Fig: 5.4. Comparison between the MCH of adult males and females from the subject and the control families. A large number of individuals from the subject families have low MCH. However, many adult females from the control group also have low MCH.

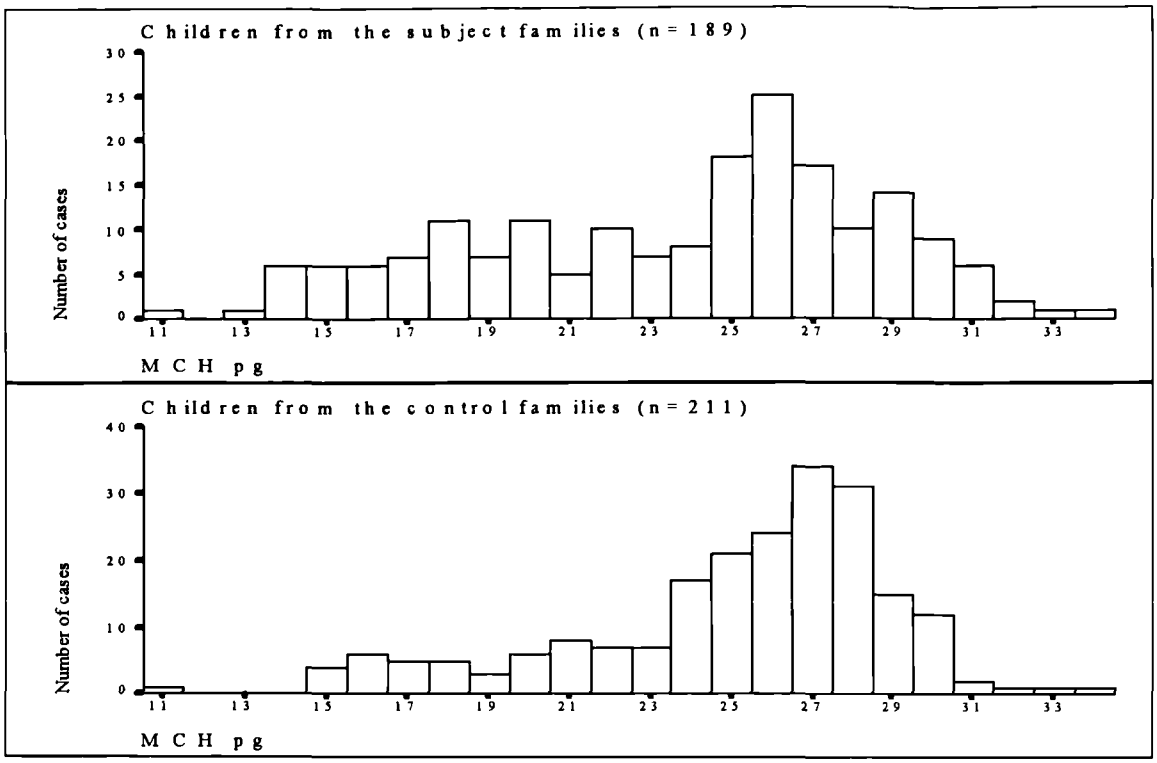


Fig: 5.5. Comparison between the MCH of children ≤15 years from the subject and the control families. A large number of children from the subject families have low MCH. However, many children in the control group also have low MCH.

Confirmation of β-thalassaemia trait:

Hb-A_2 estimation was required in 245/480 (51%) individuals in the subject families and in 24/245 (9.8%) individuals Hb-A_2 was either in the borderline range (3.0-3.4%) or their Hb-A_2 was normal but Hb was < 9 g/dl (Table: 5.6). In these individuals multiplex ARMS PCR was done to check for β-thalassaemia trait. PCR found seven new carriers (all severe mutations) that included four children less than 3 years of age and 3 adult females (Table: 5.7). They all had MCV and MCH below normal.

Number of carriers and affected children for a haemoglobin disorder:

There were 183/591 (31%) carriers for β-thalassaemia or Hb-S in the 10 subject families. The proportion of carriers in each family ranged from 20-70% (Table 5.8. and Fig: 5.6). There were 97 (29%) carriers out of 339 unmarried individuals tested in the 10 subject families (Table: 5.8). On an average there were 18 carriers per family and out of this 10 were unmarried. In addition to the carriers, there were 28 dead or alive affected children

in these families. Average number of affected children per family was 2.8 (Table 5.8). In the control families 144/397 (36%) required Hb-A_2 estimation but no carrier of β-thalassaemia or other abnormal haemoglobin was identified (Table: 5.9).

Couples at risk of a haemoglobin disorder:

Table 5.10 shows the total number of couples, their consanguinity and the number of at risk couples in the ten subject and five control families. On an average there were 22.5 couples per family. The ratio of consanguineous to non-consanguineous couples in the subjects (93/121) and the controls (56/68) did not differ significantly (p=0.09). In the ten subject families 17 couples were at risk of a haemoglobin disorder. No at risk couple was seen in the family where the index person was a thalassaemia carrier. On an average there were 1.7 at risk couples per family.

Prospective detection of at risk couples:

In the ten subject families eight at risk couples were found prospectively (Table: 5.10). In Family No: 1 three couples, in addition to the index couple, had thalassaemic children. However, only one was consanguineous (1st cousins). As a result of screening in this family some distant family members became interested and asked for testing. One such couple was found to be at risk. The couple had two children who were found to be carriers. The couple was interested in using prenatal diagnosis in future pregnancies. In Family No: 2 one couple, in addition to the index couple, was found to be at risk. This couple had one apparently normal child and the mother was six months pregnant. The father was educated and listened to the advice about the risk for his children. However, the couple declined the offer of prenatal diagnosis as the pregnancy was at an advanced stage. The child when tested after birth was found to be a carrier. The couple would be requesting prenatal diagnosis in future pregnancies. In addition to the index couples, one additional couple in Family No:5 and two additional couples in Family No: 7 had thalassaemic children. Similarly, in Family No: 10 one elderly couple, in addition to the index couple, who had grand children was found to be at risk, although they did not have an affected child. The couple was not available for detailed discussion on the deaths of

any children during early childhood. In the remaining families no other at risk couple was found.

Table: 5.6. The number of individuals who had MCV ≤75 fl or MCH ≤25 pg and required Hb-A_2 estimation or PCR for establishing the diagnosis of β-thalassaemia in the eight subject families.

Family ID:	MCV ≤75 fl or MCH ≤25 pg	β-thalassaemia trait diagnosis by:		
		Hb-A_2:	PCR:	Total:
No: 1	55/138 (39.9%)	36/55 (65.5%)	0/3	36/138 (26.1%)
No: 2	46/85 (54.1%)	23/46 (50.0%)	3/6	26/85 (30.6%)
No: 3	27/51 (52.9%)	12/27 (44.4%)	2/5	14/51 (27.6%)
No: 4	21/41 (51.2%)	16/21 (76.2%)	1/3	17/41 (41.5%)
No: 5	27/45 (60.0%)	17/27 (63.0%)	0/3	17/45 (37.8%)
No: 6	25/42 (59.5%)	18/25 (72.0%)	1/2	19/42 (45.2%)
No: 7	17/20 (85.0%)	14/17 (82.4%)	0/0	14/20 (70.0%)
No: 8	27/58 (46.6%)	12/27 (44.4%)	0/2	12/58 (20.7%)
Total:	245/480 (51.0%)	148/245 (60.4%)	7/24 (29.2%)	155/480 (32.3%)

Table: 5.7. Haematological features of seven cases of β-thalassaemia that required DNA analysis for confirmation of diagnosis.

Case:	Age: (yrs)	Sex:	Hb: (g/dl)	TRBC: ($X10^9$/L)	MCV: (fl)	MCH: (pg)	Hb-A_2: (%)	Mutation:
1.	3	F	3.5	3.23	50.8	10.8	2.0	IVSI-5 (G-C)
2.	21	F	8.2	5.99	48.6	13.7	2.4	Fr 8-9 (+G)
3.	2	M	8.5	4.81	64.2	17.7	2.1	Fr 8-9 (+G)
4.	20	F	8.4	4.61	58.6	18.2	3.0	IVSI-5 (G-C)
5.	16	F	9.3	3.80	73.7	24.5	3.1	IVSI-5 (G-C)
6.	2	M	8.5	5.29	49.5	16.1	3.2	IVSI-5 (G-C)
7.	1	M	9.2	5.30	58.1	17.4	3.0	IVSI-5 (G-C)

Table: 5.8. The result of carrier screening in 10 families with a known history of haemoglobin disorder. Family No: 1-8 are thalassaemic families and No: 9-10 are Sickle families.

Family ID:	Family members (alive):			Affected children:			Carriers:		
	Total:	Screened:	Unmarried:	Alive:	Dead:	Total:	Married:	Unmarried (%)[@]:	Total (%)[#]:
No: 1	199	138 (69.3%)	75	4	3	7	19	17/75 (22.7%)	36/138 (26.1%)
No: 2	85	85 (100%)	48	1	-	1	13	13/48 (27.1%)	26/85 (30.6%)
No: 3	55	51 (92.7%)	29	1	2	3	4	10/29 (34.5%)	14/51 (27.5%)
No: 4	48	41 (85.4%)	22	1	-	1	9	8/22 (26.4%)	17/41 (41.5%)
No: 5	69	45 (65.2%)	25	1	1	2	8	9/25 (36.0%)	17/45 (37.8%)
No: 6	60	42 (70.0%)	29	1	1	2	9	10/29 (34.5%)	19/42 (45.2%)
No: 7	80	20 (25.0%)	8	2	4	6	7	7/8 (87.5%)	14/20 (70.0%)
No: 8	98	58 (59.2%)	38	-	-	-	4	8/38 (21.1%)	12/58 (20.7%)
No: 9	79	48 (60.8%)	25	3	2	5	6	5/25 (20.0%)	11/48 (22.9%)
No: 10	73	63 (86.3%)	40	1	-	1	7	10/40 (25.0%)	17/63 (27.0%)
Total:	846	591 (69.9%)	339	15	13	28	86	97/339 (28.6%)	183/591 (31.0%)

[@] Percent of the total number of unmarried individuals screened in the family (Normal and carriers).

[#] Percent of the total number of individuals screened.

Table: 5.9. The number of individuals who had MCV ≤75 fl or MCH ≤25 pg and required Hb-A_2 estimation in the five control families.

Family ID:	**MCV ≤75 fl or MCH ≤25 pg**	**β-thalassaemia trait by Hb-A_2:**	**β-thalassaemia trait by PCR:**
No: 11	8/48 (16.7%)	None	Not done
No: 12	17/57 (29.8%)	None	Not done
No: 13	18/111 (16.2%)	None	Not done
No: 14	73/131 (55.7%)	None	Not done
No: 15	28/50 (56.0%)	None	Not done
Total:	144/397 (36.2%)	None	Not done

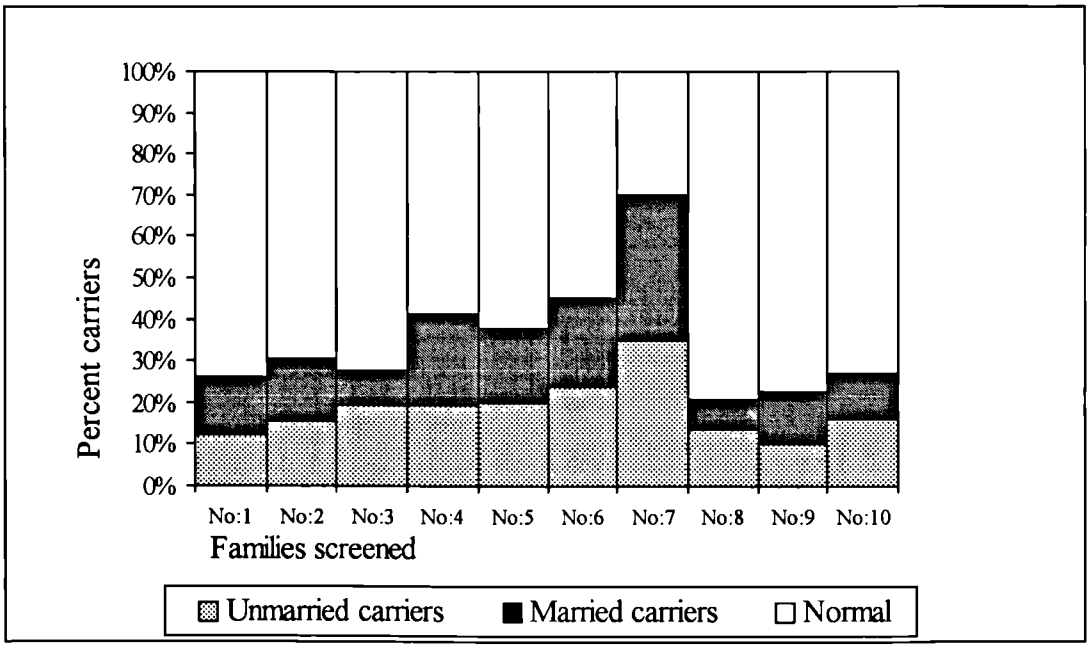


Fig: 5.6. Carrier rate for β-thalassaemia and Hb-S disorder in the 10 subject families.

Table: 5.10. Couples at risk of haemoglobin disorder in the subject and control families.

Family ID:	**Family condition:**	**Total number of couples:**			**Number of couples at risk for a haemoglobin disorder:**						
					Index:		**Prospective:**		**Total:**		
		Con:	**Non-con:**	**Total:**	**Con:**	**Non- con:**	**Con:**	**Non-con:**	**Con:**	**Non-con:**	**Total:**
Subject families:											
No: 1	β-thal major	20	32	52	-	1	1	2	1	3	4
No: 2	β-thal major	15	6	21	1	-	1	-	2	-	2
No: 3	β-thal major	8	6	14	1	-	-	-	1	-	1
No: 4	β-thal major	6	6	12	1	-	-	-	1	-	1
No: 5	β-thal major	8	11	19	1	-	1	-	2	-	2
No: 6	β-thal major	5	9	14	1	-	-	-	1	-	1
No: 7	β-thal major	14	5	19	1	-	2	-	3	-	3
No: 8	β-thal trait	8	20	28	-	-	-	-	-	-	-
No: 9	Sickle	6	14	20	-	1	-	-	-	1	1
No: 10	Sickle	3	12	15	-	1	-	1	-	2	2
Sub Total-I:	-	*93*	*121*	*214*	*6*	*3*	*5*	*3*	*11*	*6*	*17*
Control families:											
No: 11	None	13	9	22	-	-	-	-	-	-	-
No: 12	None	-	11	11	-	-	-	-	-	-	-
No: 13	None	6	17	23	-	-	-	-	-	-	-
No: 14	None	19	13	32	-	-	-	-	-	-	-
No: 15	None	18	18	36	-	-	-	-	-	-	-
Sub Total II:	-	*56*	*68*	*124*	-	-	-	-	-	-	-
Grand Total:	-	149	189	338	6	3	5	3	11	6	17

Con: consanguineous; Non-con: Non-consanguineous; Sub Total-I: Total for the subject families; Sub Total-II: Total for the control families.

Genetic counselling:

The families were approached approximately one year from the time of screening to assess their response to counselling. No contact could be established with Families No: 9 and 10 as they were residing in a very remote area. In Family No:1 three marriages took place since the completion of screening. All three were planned in the light of the results of screening. The first was between a carrier female and her normal 1st cousin. The second was between a normal male and his first cousin who was not tested and the third marriage was between a carrier female and her normal 1½ cousin. The members of the family had become very conscious about thalassaemia. In family No: 2, one marriage took place which was arranged between a male carrier and his 2nd cousin who was not tested. The reason given by the elder sister of the gentleman, who had an affected child, was that the engagement of her brother had broken three times in the past and it was with a great difficulty that they had arranged this marriage. Therefore it was not possible for them to disclose this problem. In Family No: 4 one engagement took place between two 1st cousins and it was planned in accordance with the results of screening. The boy was normal and the girl was a carrier. In Family No: 7 premarital screening was requested by two individuals who had not been screened earlier. The feedback from Family No: 3, 4, 5, and 6 was encouraging and they appeared to have become conscious of the importance of thalassaemia screening.

Discussion:

Carrier detection methods:

Screening parameters:

The results of this study suggest that screening for β-thalassaemia in most cases is straightforward. It is important to define the baseline haematological values at which thalassaemia should be suspected. The threshold values should also not miss any cases. Setting a high threshold for suspicion to include all cases, however, can be cost ineffective. The level of Hb can not be used for suspecting thalassaemia because a large proportion of women and children have low Hb due to nutritional causes. Red cell indices, on the other hand, can be extremely useful as a first line screening procedure. The cut off limit for MCV ≤75 fl and MCH ≤25 pg (Modell and Berdoukas 1984) were found

useful. Analysis of MCV and MCH in 264 β-thalassaemia carriers diagnosed by PCR (Chapter: 4) showed that only 9/264 (3.4%) cases did not fall within the defined limits. Five out of the nine such cases had Cap+1 that is a silent β-thalassaemia mutation (Cao et al, 1994). All of the Cap+1 individuals had completely normal haematology. In the remaining 4/264 (1.5%) a co-incident α-thalassaemia was suspected. Raising the threshold for MCV and MCH to any level will not identify Cap+1 carriers. It is expected that 3.4% of the carriers might be missed with the proposed cut off limit. Lowering the cut off limits for red cell indices will increase the number of Hb-A_2 estimations and the benefit gained would be only an inclusion of a small number of carriers.

Another pitfall in the proposed cut-off limit is the possibility of missing abnormal haemoglobins especially Hb-S that has normal red cell indices. The carrier rate of Hb-S in Pakistanis is 0.18% (Sharma et al, 1976). Its frequency may be higher in Pathans (Khattak and Saleem 1992b) and Baluchis (this study, chapter: 4). It may be useful to include the sickling test as a screening procedure in the high-risk ethnic groups. Hb-E is usually associated with low or borderline red cell indices (Yeo et al, 1994). Therefore it should not be missed by the proposed policy.

One tube osmotic fragility can be a simple and cost effective alternative for red cell indices as a screening method (Kattamis et al, 1981; Silvestroni and Bianco 1983). The efficacy of this procedure could not be evaluated in this study. However, a pilot study on the correlation between microcytosis and osmotic lysis of red cells in 0.36% saline in 100 unselected samples including some with low MCV showed there were 12 false positives and 4 false negatives. The false negatives were encountered in individuals who had MCV between 70-75 fl. However, the results in 50 cases of β-thalassaemia trait showed no false negative (unpublished observations).

Confirmatory tests:

Raised Hb-A_2 provides a confirmatory test for β-thalassaemia trait (Steinberg and Adams III 1991). Hb-A_2 estimation by column chromatography is technically superior than the cellulose acetate elution method (International Committee for Standardization in

Haematology 1978). In this study Hb-A_2 was measured by elution of the fraction separated by cellulose acetate electrophoresis. The method is technically easy. A study from AFIP on a comparison between indigenously prepared DEAE cellulose chromatography columns and cellulose acetate elution for Hb-A_2 estimation showed close correlation of the results (Anwar et al, 1995). The cellulose acetate method is more suitable for a Pakistani setting because it is easy to carry out and is more cost effective.

Most of the β-thalassaemia carriers in this study had clearly raised level of Hb-A_2. However, out of 172 carriers (155 in the families and 17 in pregnant women) only 9 (5.2%) had Hb-A_2 in the normal or borderline range. This included 4 children less than 3 years of age and 5 adult females (Table: 5.7). Their DNA analysis showed severe β-thalassaemia mutations. Co-incident α-thalassaemia is unlikely in these cases because such individuals commonly have normalized red cell indices and their Hb-A_2 remains in a higher range (Kanavakis et al, 1982; Rosatelli et al, 1984). In very small children Hb-A_2 level may not reach the heights seen in an adult (Steinberg and Adams III 1991). Whether such an effect can persist for three years of age is questionable. Most if not all of the nine cases may be due to concomitant iron deficiency therefore lowering the Hb-A_2 levels. Iron deficiency in a normal individual can result in a reduction of Hb-A_2 (Steinberg and Adams III 1991) and it may coexist with β-thalassaemia (Earley et al, 1990; Hinchliffe and Lilleyman 1995), a situation that is not uncommon in Pakistani women and children (Qureshi et al, 1995). Modell and Berdoukas (1984) doubt whether iron deficiency can reduce the level of Hb-A_2 to the normal range in β-thalassaemia trait.

A practical difficulty in the diagnosis of β-thalassaemia trait is caused by coincidental α-thalassaemia. This can cause considerable diagnostic difficulties in populations where the α-thalassaemia gene frequency is high (Cao et al, 1994). However, masking of β-thalassaemia trait by α-thalassaemia is not a significant problem in Pakistan (Chapter: 4).

Another diagnostic difficulty may be caused by "silent" or "mild" β-thalassaemia alleles. In this study three such alleles were identified (Table: 6.3). These include Cap+1, -88, and Hb-E. Of the three, Cap+1 is the most difficult to detect as it is associated with normal

red cell indices and Hb-A_2 level (Cao et al, 1994). Hb-E is usually associated with borderline red cell indices (Yeo et al, 1994) but can be identified by its electrophoretic mobility (Dacie and Lewis 1991). In this study only one case with -88 was encountered which had borderline red cell indices. Identification of mild thalassaemia alleles is important because when co-inherited with a severe allele can cause a syndrome of thalassaemia intermedia of variable severity (Meloni et al, 1992). A useful strategy to avoid missing "silent" or "mild" β-thalassaemia alleles in the Pakistanis would be to carry out molecular genetic analysis on those who have borderline red cell indices, particularly if their spouses are known carriers of β-thalassaemia.

A protocol for carrier detection:

Fig: 5.7 shows a suggested flow diagram for identifying β-thalassaemia carriers in Pakistanis. This is likely to identify most carriers except those with mild mutations. Stress is laid on complete analysis of individuals whose spouses are known carriers. Some unmarried or married carriers whose spouse is not available for testing might be mislabelled as normal when they may in fact be carrying a thalassaemia mutation.

The first line screening procedure may be the single tube osmotic fragility test or red cell indices. If the osmotic fragility or the red cell indices are in the normal range the individual may not require further investigations. If however, the spouse of a haematologically normal person is a carrier then the "normal" individuals would be investigated further. If the osmotic fragility test is positive or MCV $\leq$75 fl or MCH $\leq$25 pg, Hb-A_2 would be estimated. Hb-A_2 levels $\geq$3.5% would confirm the diagnosis of β-thalassaemia trait and the individual would be counselled. If Hb-A_2 is in the borderline range (3.0-3.5%) or is <3.0% and Hb is <9 g/dl multiplex ARMS PCR for the known β-thalassaemia mutations would be done.

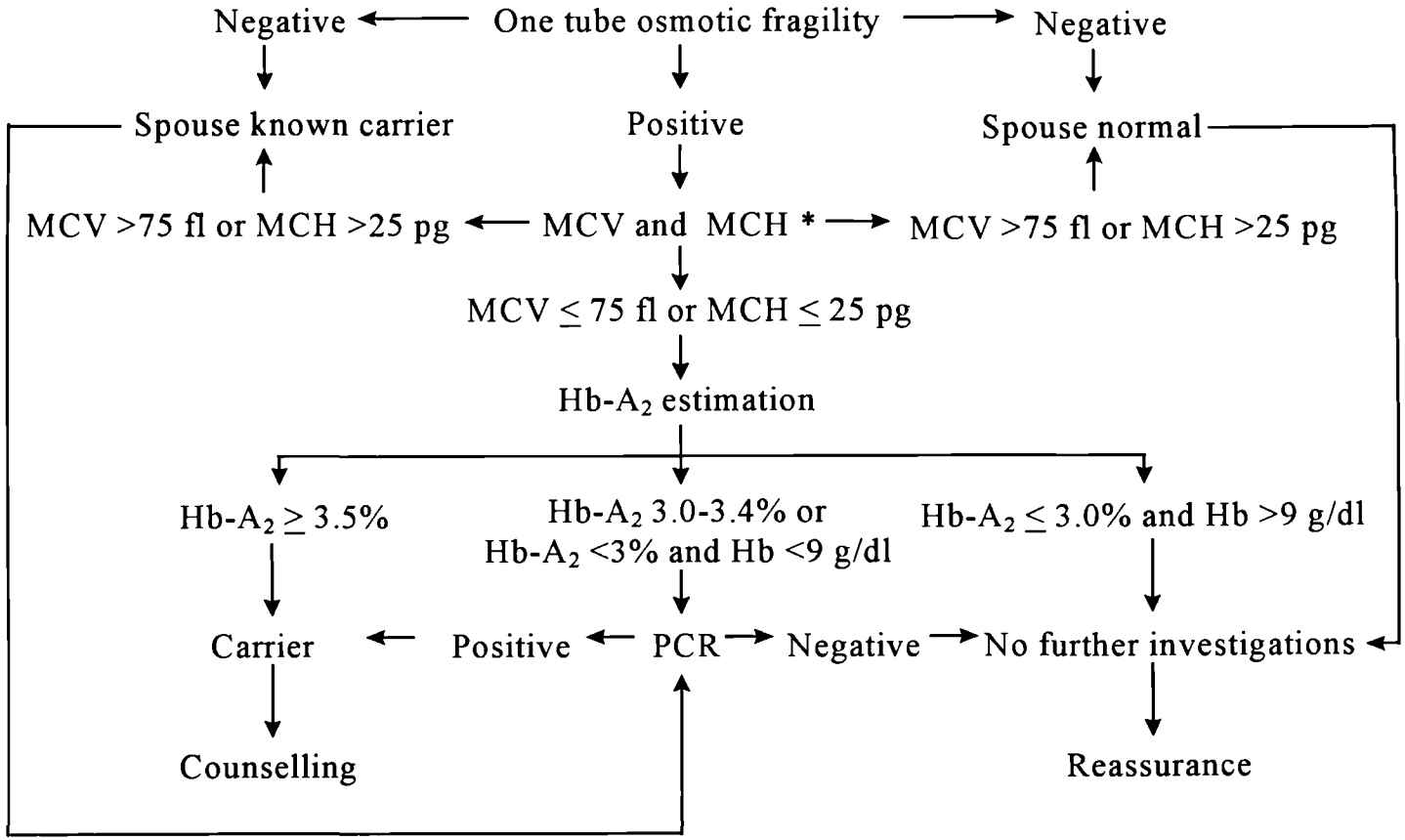


Fig: 5.7. A suggested protocol for the identification of a β-thalassaemia carrier in the Pakistani population. * Electrophoresis or Sickling test may be required in the high-risk groups.

This protocol is likely to miss abnormal haemoglobins as electrophoresis would be done only in selected cases. Since clinically significant abnormal haemoglobins are rare in Pakistan (Sharma et al, 1976), omission of electrophoresis is unlikely to have significant effects. It may be advisable to do a sickling test or electrophoresis, in the high-risk groups. In a family screening it may be advantageous to first identify the family condition by electrophoresis or DNA analysis and then screen the members accordingly.

Identification of at risk couples:

Antenatal screening:

Surprisingly, screening in 350 pregnant women in this study did not identify any at risk couple. This is probably due to a relatively small number of individuals investigated. Theoretically it is expected that at a 5% carrier rate, 1 in 400 couples would be at risk and the risk may be doubled if the spouse is a first cousin (Chapter: 9).

There are also technical difficulties in screening during pregnancy e.g. a large number of women with low MCV or MCH due to iron deficiency would require Hb-A_2 measurement in a comparatively large number of cases. Screening difficulties may also be due to an increase in MCV in pregnant β-thalassaemia carrier women by about 2% (Lewis et al, 1982; Yeo et al, 1994). Therefore the cut off limit for doing Hb-A_2 estimation will have to be increased which could increase the number of Hb-A_2 measurements required in pregnant women.

A major limitation of antenatal screening in Pakistan is that the vast majority of the pregnancies, especially in the rural areas, can not be screened as the women do not report to an antenatal clinic. Trained personnel attend only 26% of pregnant women in Pakistan (Burney 1993). Therefore it is unlikely for antenatal screening to be practically feasible.

Also the late discovery of risk in pregnancy is unlikely to lead to the abortion even if the fetus is affected. In this study only 18% of the women were seen in the first trimester, the remaining were in an advanced stage of pregnancy for prenatal diagnosis to be accepted. Another important factor that could not be assessed in this study, is the response of at risk

couples identified through antenatal screening. It is also expected that counselling would be difficult because couples who do not have an affected child may find it difficult to accept prenatal diagnosis.

Screening in the index families:

The results of screening in the index families suggest that nearly 2/3rd of the families may be willing to undergo screening. The response may be improved by more intensive efforts to pursue families. A major advantage of screening in the index families, apart from prospective detection of at risk couples, is the identification of the carriers early enough for marriage choices to be affected. The results of follow-up for one year after screening in the study families showed that there was some effect on the marriage choices. Long-term follow-up of these families, however, would be required to draw firm conclusions.

In addition to identifying a large number of carriers in a family, there are several other advantages of the targeted approach for thalassaemia screening. The process of screening can be initiated by counselling of one or a few individuals in a family. The counselling itself may be easy and effective because most family members have some knowledge of a child's illness in the family. This is a significant advantage for a community where the literacy rate is very low. There are also some technical advantages in a family screening. It can be started from the eldest available members. Their children may be screened only if one or both parents are found to be carriers. However, a practical problem may arise because in a field trip to a rural area it may be difficult to screen the elders first and then sample the children at a later stage. The one tube osmotic fragility test (Kattamis et al, 1981) could be useful for this purpose as it can indicate on the spot the members to be included or excluded from screening. The actual number of individuals that may require screening can be reduced to almost 50% or less.

Distribution of the thalassaemia gene in the community:

Autosomal recessive disorders are likely to have a uniform distribution in a randomly mating population and a clustered distribution in a population where consanguineous marriages are common (Modell and Kuliev 1992). The observations in this study also

support this hypothesis. Several families, that had a history of a haemoglobin disorder, were found to have clustering of the carriers and the homozygotes. Interestingly, there was not a single β-thalassaemia carrier in 397 individuals from the five control families. Screening in a similar number of people from the general population would have identified approximately 20 carriers. In Pakistan where consanguineous marriage is customary the autosomal recessive genes may be "trapped" in a relatively few selected families. This further supports the concept that targeting index families for screening a recessive disorder can be the most suitable and cost effective choice for screening in a Pakistani setting.

6 Characterization of β-thalassaemia mutations

Introduction:

This chapter describes study of β-thalassaemia mutations in all ethnic groups in Pakistan. In addition, data on the molecular pathology of thalassaemia intermedia is also presented.

Subjects:

A total of 712 unrelated individuals were studied. These included 184 β-thalassaemia heterozygotes, 519 β-thalassaemia homozygotes and 9 cases of Hb-S/β-thalassaemia who were also receiving transfusions. The heterozygotes were identified from individuals referred to the department of haematology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, for screening of β-thalassaemia. Their diagnosis was made by red cell indices and HbA_2 estimation. The patients with β-thalassaemia major and Hb-S/β-thalassaemia included in the study were receiving treatment at Fatimid Foundation Thalassaemia Treatment Centres, Karachi, Lahore, and Peshawar, Hussaini Blood Bank, Karachi and Pakistan Thalassaemia Welfare Society Thalassaemia Treatment Centre, Rawalpindi. The patients were diagnosed at various government and private laboratories.

At the time of sampling each individual or his/her parents were interviewed. Information including age, ethnic group, parental consanguinity and transfusion history was recorded. From each individual 3-5 ml of blood was collected in EDTA and kept refrigerated until DNA was extracted.

Thalassaemia intermedia:

Patients of β-thalassaemia who had a mild phenotype, characterized by late start or infrequent requirement of transfusions, were further investigated for the type of β-thalassaemia mutation, Xmn-I polymorphism and coincidence of α-thalassaemia ($-\alpha^{3.7}$ and $-\alpha^{4.2}$). Xmn-I

polymorphism was also investigated in 39 randomly selected patients of severe β-thalassaemia major and 58 apparently normal individuals (Controls).

Results:

Distribution of ethnic groups:

The subject's diagnosis and ethnic group is shown in Table: 6.1.

Table: 6.1. Distribution of the subjects according to ethnic group and diagnosis.

Ethnic Group:	Thalassaemia trait:	Thalassaemia major:	Hb-S/ Thalassaemia:	Total Subjects:	Total mutant chromosomes:
Punjabi:	117	139	-	256	395
Pathan:	57	78	4	139	221
Sindhi:	3	129	1	133	263
Baluchi:	6	83	1	90	174
Mohajirs:	1	90	3	94	187
TOTAL:	184	519	9	712	1240

Age distribution:

Subjects with β-thalassaemia major ranged in age from 3 months to 35 years (Fig: 6.1). Their distribution according to age was as follows: <1 year 6%, 1-2 years 9%, 2-5 years 33%, 5-10 years 34%, and >15 years 18%. The ages of patients with Hb-S/β-thalassaemia ranged from 4-17 years (Mean 9 years).

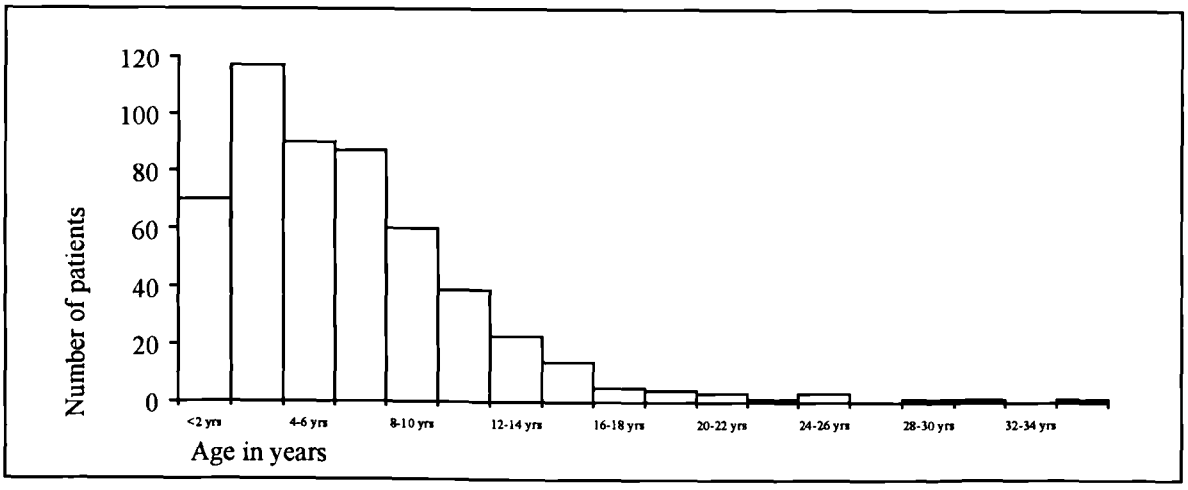


Fig: 6.1. Age distribution of 519 patients of thalassaemia major in whom mutation analysis was done.

Mutation analysis:

Each heterozygote of β-thalassaemia represented one mutant allele and homozygotes represented two alleles. In 184 heterozygotes, 519 cases of β-thalassaemia major and 9 cases of Hb-S/β-thalassaemia 1240 mutant alleles were identified.

Screening by ARMS:

The ARMS method identified 1218/1240 (98.2 %) alleles. Bright and clear bands showed a positive result (Fig: 6.2a). For most of the mutations negatives were clear negative but a faint false positive band was seen in some (Fig: 6.2b).

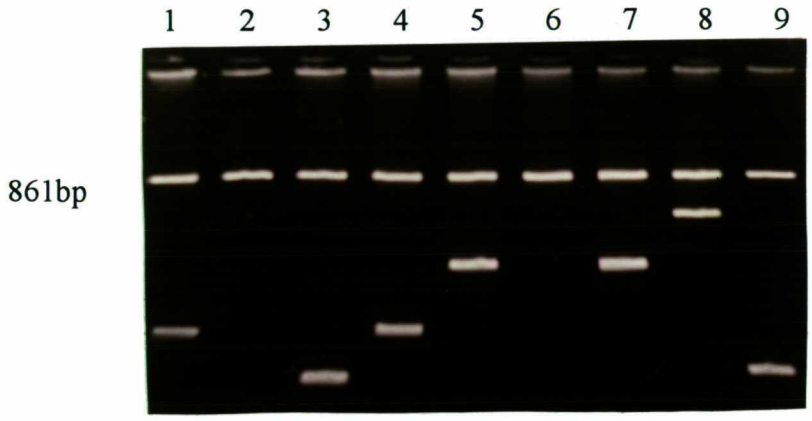


Fig: 6.2a. Ethidium bromide stained agarose gel electrophoresis of ARMS amplified products. All lanes show 861bp control bands. Lane 1 and 4 show bands of IVSI-5, lane 3 is Cd5, lane 5 and 7 are Fr 41-42, lane 8 is IVSII-1 and lane 9 is Fr 8-9.

False positive bands were always seen with Hb-E mutant and Cd 15 (G-A) normal primers. Similarly, faint positive bands, although of much less intensity, were also given by Fr 8-9 (+G) mutant, Cap+1 (A-C) mutant, and IVSI-1 (G-T) normal primers. The false positive bands could be made less intense by (a) reducing the amount of Taq polymerase from Advanced Biotechnologies, UK (b) using Taq from Perkin Elmer UK, (c) increasing the annealing temperature to 67° C instead of the 65° C (d) comparing the intensity of 861 bp control band and the mutant/normal band: the presence of a brighter control band (861 bp) suggested a false positive result. Comparing the bands for known positive or negative samples also helped to identify false positive bands. The false positive band shown by Hb-E mutant primer was

made even fainter by redesigning the primer with a (G/G) mismatch instead of the (G/A) mismatch at position -3 relative to the 3' end of the primer (Table: 6.3).

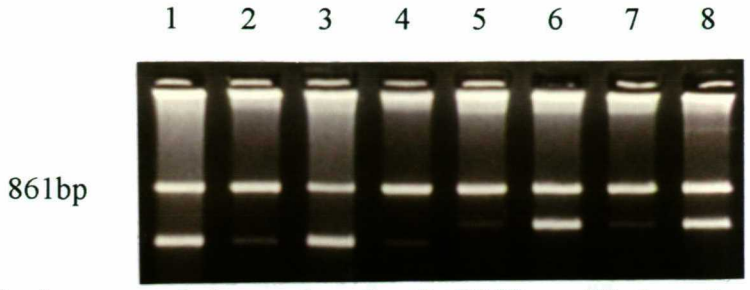


Fig: 6.2b. Agarose gel electrophoresis of ARMS amplified products for Cd15 (lanes 1 and 3) and Cap+1 (lanes 6 and 8). False positive bands for the same mutations can be seen in the lanes 2, 4, 5 and 7. 861 bp control fragments are visible in all lanes.

Twenty two alleles were not characterized by the ARMS method. Subsequent investigations (DGGE and sequencing) suggested that 8 of these were false negatives and analysis of the records showed that the negative result was a consequence of human errors. In five cases testing by ARMS had been omitted due to a clerical error and in 3 the absence of 861 bp control band, indicating failure of PCR, had been overlooked. These eight samples gave a clear positive result when subsequently tested by the respective ARMS primers i.e. Cd 15 (G-A): 3, Fr 16 (-C): 2, Cap+1 (A-C): 2, and Cd 30 (G-A): 1). This left 14/1240 (1.1%) uncharacterized mutations.

Denaturing Gradient Gel Electrophoresis (DGGE):

The β-globin gene was tested by DGGE in overlapping regions (Fig 3.1). The primers used covered the gene except the major portion of IVSII (IVSII-30 to IVSII-588), the last 14 codons, the termination site and the polyadenylation site.

Fragments I and II:

This region contains most of the promoter region sequences and the first few codons of Exon-1. The majority of mutations including the C-T polymorphism in the 2nd codon were identified by DGGE of this region (Fig: 6.3). The fast moving bands in a particular lane represented homoduplexes containing the mutation while the slower bands resulted from heteroduplex

formation between the mutant and the normal sequences. Single bands were seen when the test sample was homozygous for either the mutation/polymorphism or the normal sequence.

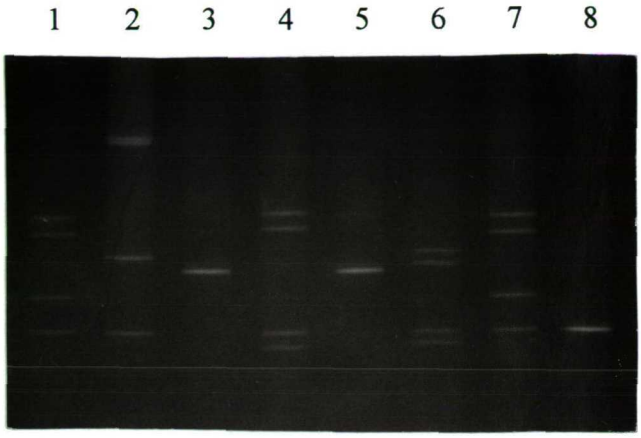


Fig: 6.3. DGGE of the amplified fragment-I after ethidium bromide staining. Lane 1 and 7 show heterozygous polymorphism and lane 8 is homozygous polymorphism at Cd2, lane 2 is heterozygous Cd15, lane 3 and 5 are homozygous Fr16, lane 4 is heterozygous Cd5 and lane 6 is heterozygous Fr 8-9.

DGGE of fragment-I revealed seven mutant alleles. Some samples were also homozygous or heterozygous for the polymorphism at the Cd2. Careful analysis of the patterns produced by the polymorphism was required to distinguish it from a mutation. The problem was overcome by including known samples with or without Cd2 polymorphism. The fragment-II was amplified by a set of primers in which the GC clamp was shifted to the downstream primer as compared to the GC clamp used with the upstream primer that amplified the fragment-I. This resulted in identification of three mutantions, all shown by subsequent sequencing to have the -88 (C-T) mutation. In fact, close proximity of -88 (C-T) to the GC clamp in fragment-I prevents identification by using this fragment.

Fragment-III:

Fragment-III was large (424bp) and encompassed promoter sequences, Exon-I and most of the IVS-I. It was found to be useful for identification of mutations located in the splice junction of Exon-I and the IVS-I. DGGE of this fragment resulted in identification of two

mutations. Subsequent sequencing revealed them to be Cd 30 (G-A), and IVSI-1 (G-A).

Fragments IV and V:

Fragments IV and V were useful for identifying mutations in Exon-II. DGGE of this fragment identified one mutation which on subsequent sequencing showed Cd 39 (C-T) which is a Mediterranean mutation.

Fragment VI:

This fragment spans the terminal part of IVSII and the major portion of Exon-III. This region contains several thalassaemia mutations and a C-T polymorphism at position IVSII-666 (Ghanem et al, 1992). DGGE of this fragment did not reveal any mutant allele. However, the polymorphism was seen on 7 out of 22 chromosomes.

Genomic sequencing:

Genomic sequencing elucidated the precise nature of the mutations identified by DGGE. The results of sequencing on single stranded as well as the double stranded templates were comparable but the double stranded protocol had the advantage of being quicker.

The results are summarised in Table: 6.2. New mutations identified by sequencing include Cd 39 (C-T), IVSI-1 (G-A) and -88 (C-T). A Pathan patient with severe transfusion dependent thalassaemia was found to be homozygous for a novel mutation in the Exon-III of β-globin gene, namely a 17 bp deletion (-TGC/AGG/CTG/CCT/ATC/AG) involving codons 126 to 131 (Fig: 6.4). The deletion was located between two copies of a CAG sequence present in Cd 125/126 and Cd 131. It was not possible to ascertain whether the breakpoint was before or following the CAG sequence in Cd 125/126. However, the Cd 125 was left intact. The deletion caused a frame-shift leading to a premature stop signal (TAA) at the new Cd 133. This deletion was not detected by DGGE because the sample had failed to amplify with the primers for the fragment VI as the downstream primer for this fragment is complementary to Cd 125-132 (Primer DG11 in Table: 3.5), deleted in this sample.

In four subjects with thalassaemia major and two with thalassaemia intermedia only one β-

thalassaemia allele was identified by ARMS and DGGE. Sequencing of the entire β-globin gene including 110 bases upstream to the Cap site and 10 bases beyond the poly-A site in all six samples could not identify the second mutant allele.

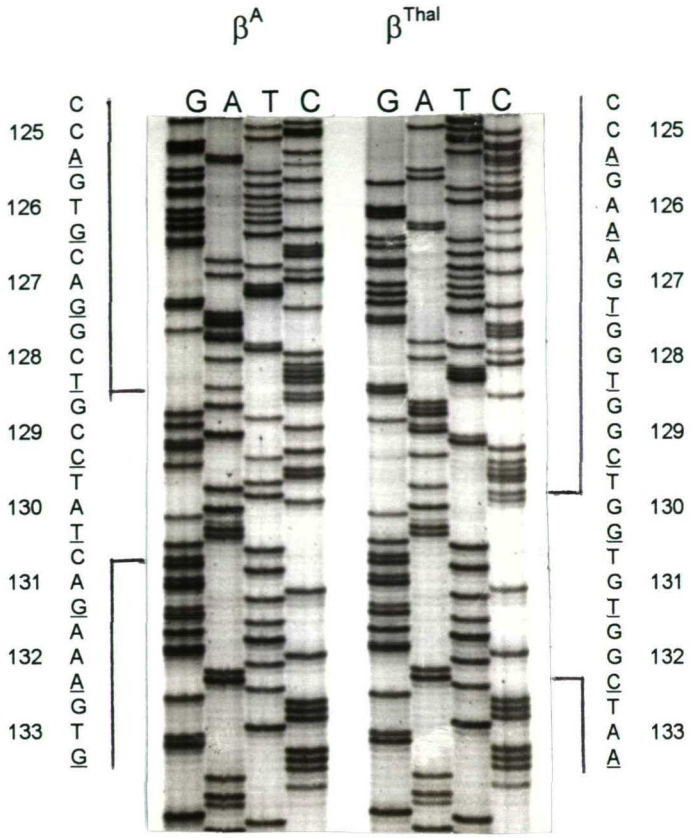


Fig: 6.4. DNA sequencing gel showing the non coding strand of exon-III containing the 17bp deletion. Sequence of the normal DNA is also shown.

Table: 6.2. Mutations characterized by genomic sequencing.

Mutations:	**Number of alleles:**	**Mutations:**	**Number of alleles:**
Cd 15 (G-A)	3	IVSI-1 (G-A)	2
Fr 16 (-C)	2	Cd 39 (C-T)	1
Cd 30 (G-A)	1	Fr 125-131 (-17 bp)	2
-88 (C-T)	3	Cap+1 (A-C)	2

Spectrum of β-thalassaemia mutations:

A total of 1225 β-thalassaemia alleles were characterized, 19 different β-thalassaemia mutations being identified (Table: 6.3). In addition, 9 alleles of Hb-S in combination with different β-thalassaemia mutations were identified. The five most frequent mutations were called common mutations. The mutations that were not common but had a frequency of 1% or more were called uncommon and those with less than 1% frequency were called rare mutations. The common mutations, IVSI-5 (G-C) (37%), Fr 8-9 (+G) (25.6%), Del 619bp (7%), Fr 41-42 (-TTCT) (6.6%), and IVSI-1 (G-T) (5%) accounted for 81% of the alleles (Table: 6.3). Six uncommon mutations formed 15% of the total and another nine (4%) were categorized as rare mutations. Six subjects with transfusion dependent anaemia had only one β-thalassaemia mutation and the other allele remained unknown. Three of these had IVSI-5 (G-C), two had Fr 8-9 (+G), and one had Fr 41-42 (-TTCT).

Punjabi:

The total of 393 alleles from Punjabi subjects included 16 different mutations (Table: 6.3). The common mutations Fr 8-9 (+G), IVSI-5 (G-C), Fr 41-42 (-TTCT), IVSI-1 (G-T) and Cd 30 (G-C) constituted 82% of the alleles. Cd 30 (G-C) replaced del 619 in the common group of mutations. The uncommon mutations formed 15.3% and rare mutations were 2.5% of the total.

Pathan:

Fr 8-9 (+G) comprised 48% of the alleles identified. The other common mutations were IVSI-5 (G-C), Fr 41-42 (-TTCT), Cd 5 (-CT), and Cd 15 (G-A) (Table: 6.3). Cd 5 (-CT) and Cd 15 (G-A) replaced IVSI-1 (G-T) and Del 619 in the common group. Two β^+ mutations Cap+1 (A-C) and -88 (C-T) comprised 4.5% of the alleles. Compound heterozygotes of Hb-S and β-thalassaemia formed 4/82 (5%) of the subjects with transfusion dependent haemoglobin disorder in Pathans. A novel 17bp deletion that involved Cd 126-131 was found in a transfusion dependent Pathan child with consanguineous parents. In twoPathan patients with apparent thalassaemia major only one β-thalassaemia allele was identified.

Sindhi:

The common mutations including IVSI-5 (G-C), del 619, IVSI-1 (G-T), Fr 8-9 (+G) and Cd 30 (G-C) comprised 88% of the alleles (Table: 6.3). Del 619 was found at a frequency of 13.6% and only one allele of IVSI minus 25 was seen. In two Sindhi patients with apparent transfusion dependent thalassaemia only one β-thalassaemia allele was identified.

Baluchi:

The mutation pattern amongst Baluchis was comparatively less heterogenous. IVSI-5 (G-C) accounted for 75% of the alleles (Table: 6.3). The other common mutations included Fr 8-9 (+G), Cd 15 (G-A) and Fr 16 (-C). There was one unidentified allele in a patient with apparent β-thalassaemia major.

Mohajir:

The pattern of mutations in Mohajirs was fairly heterogeneous (Table: 6.3). The common mutations including IVSI-5 (G-C), del 619bp, Fr 8-9 (+G), Fr 41-42 (-TTCT) and Hb-E accounted for 80% of the alleles. There were 10 (10.8%) compound heterozygotes with Hb-E/β-thalassaemia and 3 (3.2%) with Hb-S/β-thalassaemia.

Parental consanguinity and inheritance of mutations:

Both β-thalassaemia alleles were identified in 513 homozygotes. The relationship between parental consanguinity and inheritance of mutations in these patients is summarized in Table: 6.4. On the whole 315 (61.4%) were offspring of first cousin marriages, 89 (17.3%) had more distant related parents (relationship not beyond second cousin) and 109 (21.2%) had totally unrelated parents. Forty three percent of subjects whose parents were unrelated had inherited the same mutation from both the parents. It increased to 58.4% for those with more more distantly related parents, and to 88.3% for the subjects whose parents were first cousins. The pattern was generally similar in all ethnic groups (Table: 6.4). However, amongst the Baluchis, where IVSI-5 (G-C) was found at a frequency of 75%, there was no difference in the frequency of inheriting the same mutation whether the parents were consanguineous or not. In 9 Hb-S/β-thalassaemia patients 5 had unrelated parents and 4 had consanguineous parents (1st cousins).

Table: 6.3. β-thalassaemia mutations and Hb-S disorder in the ethnic groups of Pakistan.

All ethnic groups:					
Common mutations:		**Uncommon mutations:**		**Rare mutations:**	
IVSI-5 (G-C)	457 (36.9%)	Cd 15 (G-A)	51 (4.1%)	Cd 30 (G-A)	11 (0.9%)
Fr 8-9 (+G)	317 (25.6%)	Cd 30 (G-C)	43 (3.5%)	IVSII-1 (G-A)	10 (0.8%)
Del 619	85 (6.9%)	Cd 5 (-CT)	31 (2.5%)	Hb-S	9 (0.7%)
Fr 41-42 (-TTCT)	82 (6.6%)	Fr 16 (-C)	29 (2.3%)	-88 (C-T)	3 (0.3%)
IVSI-1 (G-T)	65 (5.2%)	Cap +1 (A-C)	20 (1.6%)	IVSI-1 (G-A)	2 (0.2%)
		Hb-E	13 (1.1%)	Fr 47-48 (+ATCT)	2 (0.2%)
				Fr126-131 (-17bp)	2 (0.2%)
				Cd 39 (C-T)	1 (0.1%)
				IVSI minus 25	1 (0.1%)
				Unknown	6 (0.5%)
Total 1240 (100%)	1006 (81.1%)		187 (15.1%)		47 (3.8%)
Punjabi:					
Common mutations:		**Uncommon mutations:**		**Rare mutations:**	
Fr 8-9 (+G)	146 (37.2%)	Del 619	14 (3.6%)	Cd 30 (G-A)	3 (0.8%)
IVSI-5 (G-C)	107 (27.2%)	Cd 15 (G-A)	14 (3.6%)	Hb-E	3 (0.8%)
Fr 41-42 (-TTCT)	36 (9.2%)	Cd 5 (-CT)	11 (2.8%)	Fr 47-48 (+ATCT)	2 (0.5%)
IVSI-1 (G-T)	19 (4.8%)	Cap +1 (A-C)	9 (2.3%)	IVSI-1 (G-A)	1 (0.3%)
Cd 30 (G-C)	15 (3.8%)	Fr 16 (-C)	6 (1.5%)	-88 (C-T)	1 (0.3%)
		IVSII-1 (G-A)	6 (1.5%)		
Total 393 (100%)	323 (82.2%)		60 (15.3%)		10 (2.5%)
Pathan:					
Common mutations:		**Uncommon mutations:**		**Rare mutations:**	
Fr 8-9 (+G)	105 (47.7%)	Fr 16 (-C)	8 (3.6%)	Cd 30 (G-A)	2 (0.9%)
IVSI-5 (G-C)	27 (12.3%)	Cap +1 (A-C)	8 (3.6%)	Fr126-131 (-17 bp)	2 (0.9%)
Fr 41-42 (-TTCT)	18 (8.2%)	IVSI-1 (G-T)	4 (1.8%)	-88 (C-T)	2 (0.9%)
Cd 5 (-CT)	17 (7.7%)	Del 619	4 (1.8%)	IVSII-1 (G-A)	1 (0.5%)
Cd 15 (G-A)	14 (6.4%)	Hb-S	4 (1.8%)	Cd 30 (G-C)	1 (0.5%)
				Cd 39 (C-T)	1 (0.5%)
				Unknown	2 (0.9%)
Total 220 (100%)	181 (82.3%)		28 (12.7%)		11 (5.0%)

Continued on next page.

Table: 6.3 β-thalassaemia mutations and Hb-S disorder in the ethnic groups of Pakistan. (Continued from previous page).

Sindhi:					
Common mutations:		**Uncommon mutations:**		**Rare mutations:**	
IVSI-5 (G-C)	114 (43.2%)	Fr41-42 (-TTCT)	16 (6.1%)	Hb-S	1 (0.4%)
Del 619	36 (13.6%)	Fr 16 (-C)	6 (2.3%)	IVSI minus 25	1 (0.4%)
IVSI-1 (G-T)	33 (12.5%)	Cd 15 (G-A)	5 (1.9%)	IVSI-1 (G-A)	1 (0.4%)
Fr 8-9 (+G)	29 (11.0%)			Unknown:	2 (0.8%)
Cd 30 (G-C)	20 (7.6%)				
Total 264 (100%)	232 (87.8%)		27 (10.2%)		5 (1.9%)
Baluchi:					
Common mutations:		**Uncommon mutations:**		**Rare mutations:**	
IVSI-5 (G-C)	132 (75.4%)	Fr 16 (-C)	6 (3.4%)	Fr 41-42 (-TTCT)	1 (0.6%)
Fr 8-9 (+G)	14 (8.0%)	Cd 30 (G-C)	3 (1.7%)	Cd 5 (-CT)	1 (0.6%)
Cd 15 (G-A)	9 (5.1%)	Cd 30 (G-A)	2 (1.1%)	IVSII-1 (G-A)	1 (0.6%)
		Del 619	2 (1.1%)	Hb-S	1 (0.6%)
		IVSI-1 (G-T)	2 (1.1%)	Unknown	1 (0.6%)
Total 175 (100%)	155 (88.6%)		15 (8.6%)		5 (2.9%)
Mohajir:					
Common mutations:		**Uncommon mutations:**		**Rare mutations:**	
IVSI-5 (G-C)	77 (41.0%)	Cd 15 (G-A)	9 (4.8%)	Unknown	1 (0.5%)
Del 619	29 (15.4%)	IVSI-1 (G-T)	7 (3.7%)		
Fr 8-9 (+G)	23 (12.2%)	Cd 30 (G-C)	4 (2.1%)		
Fr 41-42 (-TTCT)	11 (5.9%)	Cd 30 (G-A)	4 (2.1%)		
Hb-E	10 (5.3%)	Fr 16 (-C)	3 (1.6%)		
		Cap +1 (A-C)	3 (1.6%)		
		Hb-S	3 (1.6%)		
		Cd 5 (-CT)	2 (1.6%)		
		IVSII-1 (G-A)	2 (1.1%)		
Total 188 (100%)	150 (79.8%)		37 (19.7%)		1 (0.5%)

Table: 6.4. Relationship between parental consanguinity and inheritance of thalassaemia mutations in patients where both of the β-thalassaemia genes were identified.

Ethnic Groups (n):	1st cousins			Related but not 1st cousins			Unrelated		
	Same mutation	Different mutations	Total	Same mutation	Different mutations	Total	Same mutation	Different mutations	Total
Punjabi (137)	81 (83.5%)	16 (16.5%)	97	15 (71.4%)	6 (28.6%)	21	10 (52.6%)	9 (47.40%)	19
Pathan (75)	42 (93.3%)	3 (6.7%)	45	4 (30.8%)	9 (69.2%)	13	7 (41.2%)	10 (58.8%)	17
Sindhi (128)	77 (90.6%)	8 (9.4%)	85	10 (55.6%)	8 (44.4%)	18	7 (28.0%)	18 (72.0%)	25
Baluchi (83)	54 (87.1%)	8 (12.9%)	62	11 (84.6%)	2 (15.4%)	13	7 (87.5%)	1 (12.5%)	8
Mohajir (90)	24 (92.3%)	2 (7.7%)	26	12 (50.0%)	12 (50.0%)	24	16 (40.0%)	24 (60.0%)	40
All groups (513)	278 (88.3%)	37 (11.7%)	315	52 (58.4%)	37 (41.6%)	89	47 (43.1%)	62 (56.9%)	109

Thalassaemia Intermedia (TI):

In the 519 patients of β-thalassaemia on transfusions 39 (7.5%) had a mild phenotype characterised by a late start and less frequent requirement of blood transfusions. The Xmn-I polymorphism, type of mutations, and coincidence of α-thalassaemia are presented in Table: 6.5. In 24/39 (61.5%) patients a definite cause for mildness of the disease was identified. Xmn-I +/+ genotype was found in 14 patients, β^+-mutations with or without coincident α-thalassaemia were found in 12 patients, and coincident α-thalassaemia was found in 2 patients. Two patients had unidentified mutations in combination with IVSI-5. In 14 patients Xmn-I -/+ genotype was found. However, two such patients had Cap+1 and a suspected unknown mutation outside the β-globin gene. In the remaining 12 patients Xmn-I -/+ alone or a suspected coincidental α-thalassaemia was the likely cause of thalassaemia intermedia.

Age distribution:

The ages of TI patients ranged from 3-35 years with mean age of 12 years (Fig: 6.5). The average age at first transfusion ranged from 3-26 years (mean 7 years) (Fig: 6.6). Mean age at first transfusion when examined in relation to the underlying cause of thalassaemia intermedia (Table: 6.6), it was a maximum of 10 years in patients who either had confirmed coincidence of α-thalassaemia or in whom it was suspected. It was 6 years in patients with Xmn-I +/+ genotype and 3 years in patients with a β^+-mutation.

Table: 6.5. Molecular basis of Thalassaemia Intermedia in the ethnic groups of Pakistan.

Sr. No	Age: (yrs)	First Trans#	β-thalassaemia Mutations	Xmn-I genotype	α-Thal genotype*	Probable cause of Thalassaemia Intermedia
Punjabi:						
1	3 yrs	None	IVSI-5 (G-C)/ IVSI-5 (G-C)	+/+	αα/αα	Xmn-I +/+
2.	5 yrs	3 yrs	IVSI-5 (G-C)/ IVSI-5 (G-C)	+/+	αα/αα	Xmn-I +/+
3.	12 yrs	4½ yrs	IVSI-5 (G-C)/ IVSI-5 (G-C)	+/+	αα/αα	Xmn-I +/+
4.	12 yrs	3½ yrs	IVSI-1 (G-T)/ IVSI-1 (G-T)	+/+	αα/αα	Xmn-I +/+
5.	5½ yrs	3 yrs	IVSI-1 (G-T)/ IVSI-1 (G-T)	+/+	αα/αα	Xmn-I +/+
6.	7 yrs	5½ yrs	IVSI-1 (G-T)/ IVSI-1 (G-T)	+/+	αα/αα	Xmn-I +/+
7.	6 yrs	5 yrs	IVSII-1 (G-A)/IVSII-1 (G-A)	+/+	αα/αα	Xmn-I +/+
8.	8 yrs	5 yrs	IVSII-1 (G-A)/IVSII-1 (G-A)	+/+	αα/αα	Xmn-I +/+
9.	8 yrs	4½ yrs	Cd 30 (G-C)/Cd 30 (G-C)	+/+	αα/αα	Xmn-I +/+
10.	13 yrs	3½ yrs	IVSI-5 (G-C)/Cap+1 (A-C)	-/-	αα/αα	β^+ thal mutation
11.	5½ yrs	2 yrs	IVSI-5 (G-C)/Cap+1 (A-C)	-/-	αα/αα	β^+ thal mutation
12	6 yrs	3 yrs	IVSI-5 (G-C)/Cap+1 (A-C)	-/-	αα/αα	β^+ thal mutation
13.	13 yrs	5 yrs	Fr 8-9 (+G)/Cap+1 (A-C)	-/-	$-\alpha^{3.7}\alpha/\alpha\alpha$	β^+ thal mutation & $-\alpha^{3.7}$
14.	10 yrs	None	Fr 8-9 (+G)/ IVSII-1 (G-A)	-/+	?	Xmn-I -/+, Coincident α-thal ??
15.	7 yrs	3 yrs	IVSI-5 (G-C)/Fr 8-9 (+G)	-/-	$-\alpha^{3.7}\alpha/-\alpha^{3.7}\alpha$	Coincident α-thalassaemia
16.	20 yrs	15 yrs	Fr 47-48 (-ATCT)/Fr 47-48	-/-	$-\alpha^{3.7}\alpha/-\alpha^{3.7}\alpha$	Coincident α-thalassaemia
Pathan:						
17.	35 yrs	24 yrs	IVSI-1 (G-T)/IVSI-1 (G-T)	+/+	αα/αα	Xmn-I +/+ & α-thal (nd ??)$
18.	5 yrs	2½ yrs	IVSI-5 (G-C)/Cap+1 (A-C)	-/+	αα/αα	Xmn-I -/+, β^+ thal mutation
19.	15 yrs	3 yrs	IVSI-5 (G-C)/Cap+1 (A-C)	-/-	αα/αα	β^+ thal mutation
20.	5 yrs	2½ yrs	Cd 5 (-CT)/Cap+1 (A-C)	-/-	αα/αα	β^+ thal mutation
21.	23 yrs	17 yrs	Fr 8-9 (+G)/-88 (C-T)	-/-	αα/αα	β^+ thal & α-thal (nd ??)$
Sindhi:						
22.	18 yrs	3½ yrs	IVSI-5 (G-C)/Cd 30 (G-C)	+/+	?	Xmn-I +/+
23.	13 yrs	5 yrs	IVSI-1 (G-T)/del 619 bp	-/+	?	Xmn-I -/+,Coincident α-thala ??
24.	15 yrs	5 yrs	IVSI-5 (G-C)/Cd 30 (G-C)	-/+	?	Xmn-I -/+,Coincident α-thala ??
25.	16 yrs	9 yrs	IVSI-5 (G-C)/IVSI-5 (G-C)	-/-	?	Coincident α-thala ??
26.	15 yrs	12 yrs	IVSI-5 (G-C)/IVSI-5 (G-C)	-/+	?	Xmn-I -/+,Coincident α-thala ??
Baluchi:						
27.	16 yrs	7 yrs	IVSI-5 (G-C)/IVSII-1 (G-A)	-/+	?	Xmn-I -/+,Coincident α-thala ??
28.	11 yrs	10 yrs	IVSI-5 (G-C)/?	-/+	?	Unknown mutation ??
Mohajir:						
29.	9 yrs	5 yrs	IVSI-5 (G-C)/IVSI-5 (G-C)	+/+	?	Xmn-I +/+
30.	30 yrs	9 yrs	IVSI-5 (G-C)/IVSI-5 (G-C)	+/+	?	Xmn-I +/+
31.	17 yrs	6 yrs	IVSII-1 (G-A)/IVSII-1 (G-A)	+/+	?	Xmn-I +/+
32.	26 yrs	12 yrs	IVSI-5 (G-C)/Hb-E	-/+	?	Xmn-I -/+, β^+-mut & α-thal ??
33.	21 yrs	18 yrs	IVSI-5 (G-C)/Hb-E	-/+	?	Xmn-I -/+, β^+-mut & α-thal ??
34.	15 yrs	9 yrs	IVSI-5 (G-C)/Hb-E	-/+	?	Xmn-I -/+, β^+-mut & α-thal ??
35.	7 yrs	None	Cd 15 (G-A)/Cap+1 (A-C)	-/-	?	Xmn-I -/+, β^+-mut & α-thal ??
36.	21 yrs	5 yrs	IVSI-5 (G-C)/del 619 bp	-/+	?	Xmn-I -/+, Coincident α-thal ??
37.	31 yrs	26 yrs	IVSI-5 (G-C)/IVSI-5 (G-C)	-/+	?	Xmn-I -/+, Coincident α-thal ??
38.	6 yrs	5½ yrs	IVSI-5 (G-C)/IVSI-5 (G-C)	-/+	?	Xmn-I -/+, Coincident α-thal ??
39.	14 yrs	5 yrs	IVSI-5 (G-C)/?	-/+	?	Unknown mutation ??

Age at first transfusion; * α-thalassaemia screening included $-\alpha^{3.7}$ and $-\alpha^{4.2}$ kb deletions; $α-thal (nd): non-deletional α-thalassaemia.

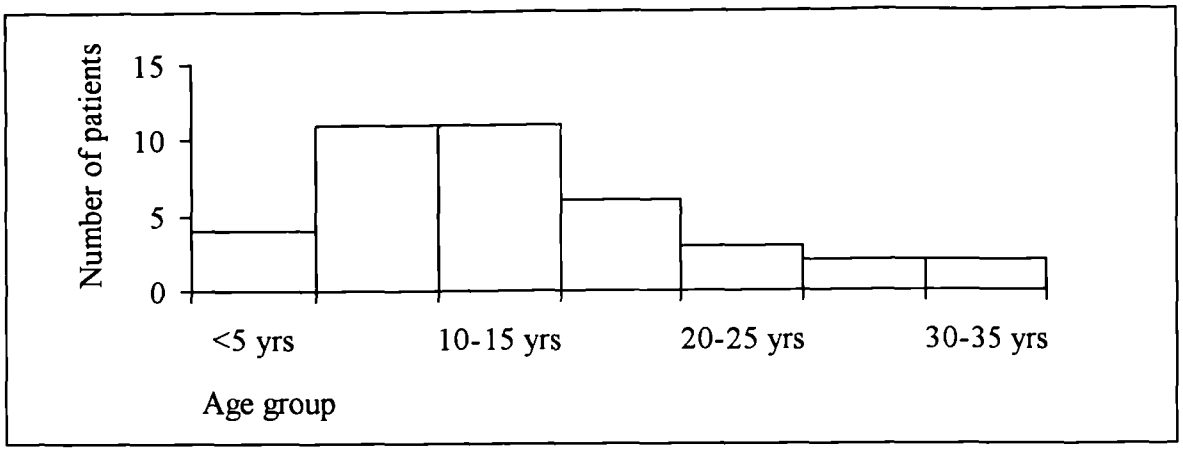


Fig: 6.5. Age distribution of 39 patients of thalassaemia Intermedia.

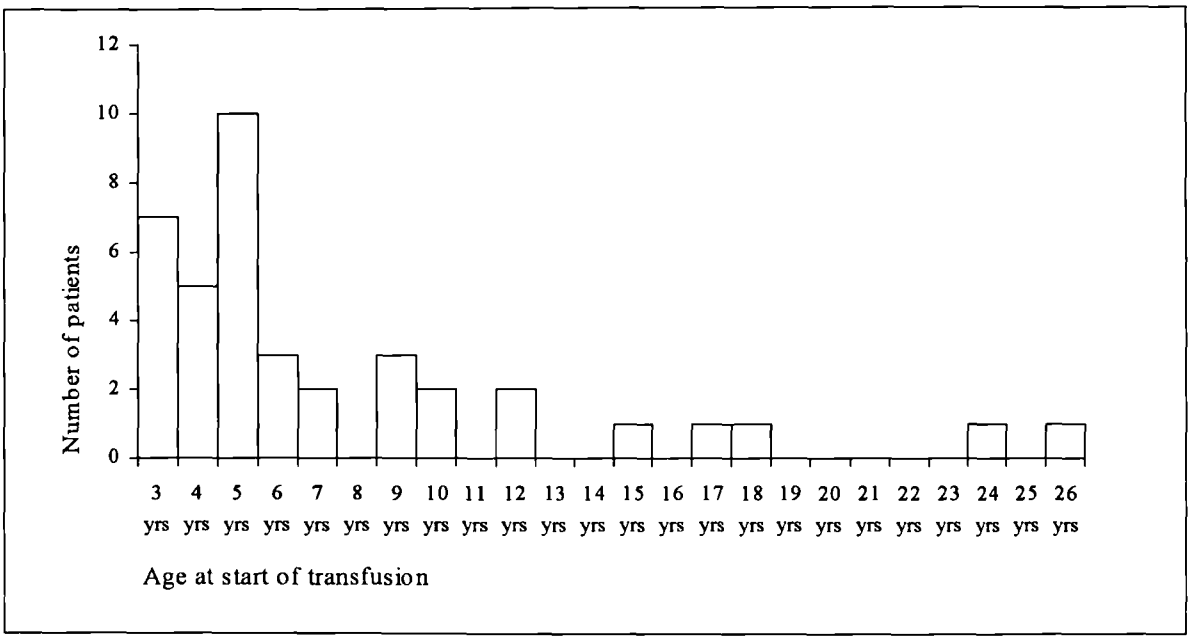


Fig: 6.6. Age distribution of 39 thalassaemia intermedia patients at the start of transfusion.

Table: 6.6. Average age at 1st transfusion and the time of examination in relation to the underlying cause of thalassaemia intermedia.

Cause of Thalassaemia Intermedia:	**n:**	**Mean age:**	
		At 1st transfusion:	**At examination:**
Xmn-I +/+ genotype	14	6 years	13 years
β^+-mutation	6	3 years	8 years
β^+-mutation and suspected coincident α-thal	6	11¼ years	18 years
Unidentified thalassaemia mutation	2	7½ years	12½ years
Confirmed coincident α-thalassaemia	2	9½ years	13½ years
Suspected coincident α-thalassaemia	9	9½ years	16 years
Total	39	7 years	14 years

Xmn-I genotype:

Xmn-I +/+ genotype accounted for thalassaemia intermedia in 14/39 (36%) patients. It was also the commonest cause for the mild phenotype in Punjabi patients where 9/16 (56%) had +/+ genotype. Xmn-I +/+ genotype was relatively uncommon in the other ethnic groups. A comparison of the Xmn-I +/+ genotype between patients of thalassaemia intermedia, severe β-thalassaemia major and normal individuals showed significant differences (Table: 6.7). There was a marked difference between thalassaemia intermedia and thalassaemia major (p=0.0005). The difference was less marked between thalassaemia intermedia and normal individuals (p=0.046) and between normal individuals and thalassaemia major (p–0.024). Xmn-I -/+ genotype was observed in 13/39 (33.3%) patients of thalassaemia intermedia. However, 9/39 (23%) patients of thalassaemia major also had Xmn-I -/+ genotype (Table: 6.8).

Correlation between the mutations and the Xmn-I genotypes in patients of thalassaemia intermedia (Table: 6.5) and thalassaemia major (Table: 6.8) showed that association between some mutations and the '+' site was present (Table: 6.9). IVSI-5 (G-C), for example, was definitely associated with '+' site in 12/33 alleles in thalassaemia intermedia and 1/25 alleles in thalassaemia major. Its association was suspected in 9/33 alleles in thalassaemia intermedia and 3/25 alleles in thalassaemia major patients. IVSI-1 (G-T), IVSII-1 (G-A) and Cd 30 (G-C) were other mutations that showed association with the "+" site.

Table: 6.7. A comparison of the Xmn-I genotypes in patients of thalassaemia intermedia, thalassaemia major and normal individuals.

Xmn-I genotype:	Thal Intermedia:	Thal Major :	Normal:
-/-	12 (30.8%)	30 (76.9%)	30 (51.7%)
-/+	13 (33.3%)	9 (23.1%)	20 (34.5%)
+/+	14 (35.9%)	None	8 (13.8%)
Total:	39 (100%)	39 (100%)	58 (100%)

Table: 6.8. β-thalassaemia and Xmn-1genotypes in patients of thalassaemia major.

Mutation:	Xmn-1 genotype:	Mutation:	Xmn-1 genotype:	Mutation:	Xmn-1 genotype:
IVSI-5/IVSI-5	-/-	IVSI-5/Fr 8-9	-/-	Fr 8-9/IVSII-1	-/+
Fr 41-42/Fr 41-42	-/-	Fr 8-9/Hb-E	-/-	IVSI-5/Hb-E	-/+
Fr 41-42/Fr 41-42	-/-	Fr 8-9/Fr 8-9	-/-	IVSI-5/IVSII-1	-/+
IVSI-5/IVSI-5	-/-	Fr 8-9/Fr 8-9	-/-	IVSI-5/Cap+1	-/-
Fr 41-42/Cd 5	-/-	Fr 8-9/IVSI-5	-/-	IVSI-1/del 619	-/+
IVSI-5/IVSI-5	-/-	Cd 15/Cd15	-/-	Cd 5/Cap+1	-/-
Del 619/Cd 5	-/-	IVSI-5/IVSI-5	-/-	Fr 8-9/Cap+1	-/-
Fr 41-42/Fr 41-42	-/-	IVSI-5/IVSI-5	-/-	Fr 8-9/Fr 8-9	-/-
IVSI-5/IVSI-5	-/+	IVSI-5/Cd 15	-/+	IVSI-5/Cap+1	-/-
Fr 8-9/Fr 8-9	-/-	IVSI-5/IVSI-5	-/-	Cd 15/Cd 15	-/+
Fr 8-9/Fr 8-9	-/-	Fr 8-9/Fr 41-42	-/+	Fr 41-42/Fr 41-42	-/-
Fr 41-42/Cd 15	-/+	IVSI-5/IVSI-5	-/-	Fr 8-9/Fr 41-42	-/-
Fr 8-9/Fr 8-9	-/-	Fr 8-9/Fr 8-9	-/-	IVSI-5/IVSI-5	-/-

Table: 6.9. Association between Xmn-I "+" site and β-thalassaemia mutations in patients of thalassaemia major and thalassaemia intermedia.

Mutation:	Thalassaemia major:				Thalassaemia Intermedia:			
	-	+	+?*	Total:	-	+	+?*	Total:
IVSI-5 (G-C)	21	1	3	25	12	12	9	33
Fr 8-9 (+G)	20	-	1	21	3	-	1	4
Fr 41-42 (-TTCT)	10	-	2	12	-	-	-	-
IVSI-1 (G-T)	-	-	1	1	-	8	1	9
IVSII-1 (G-A)	-	-	2	2	-	6	2	8
Cd 15 (G-A)	3	1	2	6	1	-	-	1
Cd 30 (G-C)	-	-	-	-	-	3	1	4
Cap +1 (A-C)	4	-	-	4	7	-	1	8
Cd 5 (-CT)	3	-	-	3	1	-	-	1
Hb-E	1	-	1	2	-	-	3	3
Del 619	1	-	1	2	1	-	1	2
Others	-	-	-	-	3	-	2	5
Total:	63	2	13	78	28	29	21	78

* Suspected "+"

Type of β-thalassaemia mutations:

Table: 6.10 gives a comparison between the frequency of mutations in thalassaemia major and thalassaemia intermedia patients. Significant differences in the frequency of Fr 8-9, IVSI-1 (G-T), Cap+1 and IVSII-1 were observed between the two groups. Similarly, differences in the frequency of Fr 8-9, Del 619, IVSI-1 (G-T) and Cd 5 were also observed between the younger (<3 years) and the older (≥3 years) patients of thalassaemia major.

There were 12/39 (31%) patients who had mild β-thalassaemia mutations in combination with another β^{o} or β^{+}-mutation. These included Cap+1 (8), Hb-E (3) and -88 (1). Out of 519 cases of homozygous β-thalassaemia 17 had Cap+1 mutation in combination with another severe β-thalassaemia mutation. However, their transfusion dependency was variable. Nine patients started transfusions during the first year of life while 3 received their first transfusion at or around three years of age and five received the first transfusion at an average age of 10 years. In the later group of five patients one had a coincident α-thalassaemia ($-\alpha^{3.7}\alpha/\alpha\alpha$) while in the other four although α-thalassaemia could not be shown due to technical reasons, it was suspected because no apparent cause for the mild phenotype was seen.

There were 10/519 (1.9%) patients who had Hb-E in combination with a β-thalassaemia mutation. Three of these patients had thalassaemia intermedia (mean age at first transfusion 13 years) while all others had severe thalassaemia phenotype (mean age at first transfusion 1½ years). In the former three patients coincident α-thalassaemia was suspected.

In two patients with thalassaemia intermedia only one β-thalassaemia allele (IVSI-5) was identified and the other allele could not be found in the β-globin gene.

Co-incident α-thalassaemia:

Effect of α-thalassaemia could not be investigated in samples where DNA was extracted without the use of Proteinase-K, as the quality of DNA was not good for PCR based determination of α-thalassaemia. In 22 patients, in whom it was possible to test for α-thalassaemia deletions, 2 had $-\alpha^{3.7}\alpha/ -\alpha^{3.7}\alpha$ genotype. One patient of thalassaemia intermedia, who also had the Cap+1 (mild β^{+}-mutation), had $-\alpha^{3.7}\alpha/\alpha\alpha$ genotype. The phenotype of this patient was less severe as compared to the other patients with the Cap+1 mutation.

Screening for α-thalassaemia in 100 randomly selected patients of thalassaemia major found five patients with $-\alpha^{3.7}\alpha/\alpha\alpha$ genotype (Chapter 4). All five had severe β-thalassaemia mutations but none had an intermedia phenotype. In 13 patients coincident α-thalassaemia (deletional or non-deletional) was suspected.

Table: 6.10. Comparison of β-thalassaemia mutations in patients with thalassaemia major and thalassaemia intermedia.

Mutation:	**All patients:**	**Thalassaemia major (TM):**			**Thalassaemia Intermedia (TI):**	***p* value:**	
		< 3 years:	**≥ 3 years:**	**All:**		**TM: < 3 years versus ≥ 3 years:**	**TI vs TM:**
IVSI-5	413 (39.8%)	97 (36.7%)	283 (40.7%)	380 (39.6%)	33 (42.3%)	-	-
Fr 8-9	236 (22.7%)	87 (33.0%)	146 (21.0%)	233 (24.3%)	3 (3.8%)	0.003	0.0003
Del 619	80 (7.7%)	12 (4.5%)	66 (9.5%)	78 (8.1%)	2 (2.6%)	0.01	-
Fr 41-42	67 (6.5%)	15 (5.7%)	52 (7.5%)	67 (7.0%)	-	-	-
IVSI-1 (G-T)	57 (5.5%)	5 (1.9%)	43 (6.2%)	48 (5.0%)	9 (11.5%)	0.009	0.025
Cd 15	40 (3.9%)	13 (4.9%)	25 (3.6%)	38 (4.0%)	2 (2.6%)	-	-
Cd 30 (G-C)	39 (3.8%)	6 (2.3%)	29 (4.2%)	35 (3.6%)	4 (5.1%)	-	-
Fr 16	26 (2.5%)	10 (3.8%)	16 (2.3%)	26 (2.7%)	-	-	-
Cd 5	18 (1.7%)	11 (4.2%)	6 (0.9%)	17 (1.8%)	1 (1.3%)	< 0.0001	-
Cap+1	17 (1.6%)	4 (1.5%)	5 (0.7%)	9 (0.9%)	8 (10.3%)	-	<0.0001
Hb-E	11 (1.1%)	1 (0.4%)	7 (1.0%)	8 (0.8%)	3 (3.8%)	-	-
IVSII-1	10 (1.0%)	1 (0.4%)	1 (0.1%)	2 (0.2%)	8 (10.3%)		<0.0001
Cd 30 (G-A)	9 (0.9%)	2 (0.8%)	7 (1.0%)	9 (0.9%)	-	-	-
-88	3 (0.3%)	-	2 (0.3%)	2 (0.2%)	1 (1.3%)	-	-
IVSI-1 (G-A)	2 (0.2%)	-	2 (0.3%)	2 (0.2%)	-	-	-
Fr 47-48	2 (0.2%)	-	-	-	2 (2.6%)	-	-
Cd 126-132	2 (0.2%)	-	2 (0.3%)	2 (0.2%)	-	-	-
Unknown	6 (0.6%)	-	4 (0.9%)	4 (0.4%)	2 (2.6%)	-	-
Total:	1038 (100%)	264 (100%)	696 (100%)	960 (100%)	78 (100%)	-	-

Discussion:

This study provides a basis for characterizing β-thalassaemia mutations in Pakistan. In the subsequent sections the choice of method and the screening strategy for mutation detection will be discussed.

Choice of method for mutation analysis:

Three different methods were used: ARMS, DGGE and genomic sequencing. Most alleles were identified by the ARMS method, which is quick, sensitive and specific for identifying known β-thalassaemia mutations. False positive results may be overcome by modifying the procedure e.g. redesigning primers, and reducing the amount of Taq polymerase. Comparison with known positive or negative samples and careful comparison of the amplification of control and mutant/normal sequences can further reduce the risk of error. In ARMS PCR the mutant/normal sequences are smaller and are preferentially amplified relative to the 861bp control fragment so a true positive band is always brighter than the control and a false positive band is less bright.

Carry-over from one sample to another leading to false positive results is avoided by careful procedures: using separate pipettes for pre and post amplification steps, avoiding splashes, and using positive displacement pipettes (Kwok and Higuchi 1989). Use of uracil DNA glycosylase to control carry over contamination in PCR can also be useful (Longo et al, 1990). Occasionally, extraneous DNA may contaminate PCR reagents whose presence can be excluded by using reagent blanks i.e. PCR run without adding DNA.

Denaturing Gradient Gel Electrophoresis (DGGE) provides information about the presence of a mutation but it gives little information about its nature (Myers et al, 1987). DGGE is extremely useful as the first line investigation for locating unknown sequence changes in the genome (Ghanem et al, 1992). To use DGGE alone for characterizing mutations requires each batch of samples to include appropriate controls for all the mutations and polymorphisms being investigated (Cai and Kan 1990). However, DGGE is labour intensive and less sensitive than ARMS, is unable to identify mutations very close to the primer with the GC clamp and polymorphisms can cause difficulty in interpreting results. The polymorphism present in the

second codon is particularly important, as many β-thalassaemia mutations are located in its vicinity and the same mutation gives different results in the presence or absence of this polymorphism.

Direct sequencing on PCR-amplified DNA is the standard method for identifying unknown sequences (Engelke et al, 1988; Rao 1994). A single stranded DNA template is used in most conventional sequencing protocols. The template may be prepared by asymmetric PCR (Varawalla et al, 1991b) or by magnetic particle separators (Hultman et al, 1989; Thein and Hinton 1994), but this is time consuming. In this study sequencing of double stranded and single stranded DNA templates gave comparable results. The later has the advantage of being simple and quick.

Spectrum of thalassaemia mutations:

Most studies of molecular genetics of thalassaemia on the Indian Subcontinent have been done on selected individuals settled in the Western countries (Kazazian et al 1990; Varawalla et al, 1991). These studies are inadequate because of the lack of representation of the ethnic groups. The present study of over 1200 mutant alleles provides a comprehensive picture of β-thalassaemia mutations in Pakistan.

The overall spectrum of mutations in Pakistan is fairly heterogenous and 19 different mutations were identified. The five common mutations, IVSI-5 (G-C) (37%), Fr 8-9 (+G) (25%), del 619bp (7%), Fr 41-42 (-TTCT) (7%), and IVSI-1 (G-T) (5%) account for 81% of the alleles. In each ethnic group four or five common mutations account for over 80% of the alleles. The spectrum of mutations is heterogenous in all of the ethnic groups except in Baluchis where IVSI-5 alone comprises 75% of the alleles.

Geographical and ethnic differences in the prevalence of mutations:

The spectrum of mutations in almost all ethnic groups in this study is fairly heterogenous. However, the spectrum in particular is more heterogenous in the Punjabis and the Pathans that mainly reside in the northern parts of the country. Several factors e.g. time since appearance of a mutation, population migrations, and random genetic drift might have contributed to the

genetic heterogeneity (Bodmer and Cavalli-Sforza 1976).

The effect of time since appearance of a mutation:

IVSI-5 (G-C) and Fr 8-9 (+G) are consistently the most common mutations through out Pakistan. Several studies have shown that IVSI-5 (G-C) is the most prevalent mutation on the Indian subcontinent (Varawalla et al, 1991a), Burma (Brown et al, 1992), Iran and the UAE (Quaife et al, 1994). The wide distribution of IVSI-5 (G-C) indicates that it is probably the oldest and the first mutation to appear in this region. IVSI-5 (G-C) is also associated with several haplotypes as compared to other mutations (Varawalla et al, 1992). This suggests that sufficient time has elapsed since its first appearance to allow several meiotic recombination events creating more than one haplotype. Alternatively, IVSI-5 (G-C), like Cd39 (C-T) in the Mediterranean (Pirastu et al, 1987), might have arisen at more than one occasion, each time on a new haplotype. Fr 8-9 (+G) is also widely distributed in the northern region of Pakistan and could have been one of the first mutations to appear in this region. It appears that once the most common mutations had established themselves in a state of balanced polymorphism, widespread propagation of the subsequently developing or arriving mutations was prevented by the already "established" gene frequencies of the common mutations (Modell and Berdoukas 1984).

Effect of population migration:

New mutations may be brought in to an area through migration of populations. It is likely that three typical Mediterranean mutations (Cd39 (C-T), IVSI-1 (G-A), and IVSII-1 (G-A) observed in some subjects from the northern Pakistan arrived through population migrations as invaders from Central Asia, Iran, Turkey and Greece have all had their influence on this area (Halliday 1994). Hb-E amongst the Mohajirs, many of whom migrated to Pakistan from the province of Bihar in the eastern part of India, is also the result of population migration.

Previous studies found the 619 bp deletion in over 50% of β-thalassaemia alleles in Sindhis (Thein et al, 1984, Varawalla et al, 1991a), but in this study it accounted for only 14% of the alleles in Sindhis. The higher prevalence in previous reports was probably due to selected sampling. Del 619 was also seen quite frequently in Mohajirs who also include people of Gujrati descent among whom the 619bp deletion is common (Varawalla et al, 1991a).

In this study Baluchis were found to have the least heterogeneous pattern of mutations and IVSI-5 (G-C) accounted for 75% of the alleles. The history of the Baluchis dates back to 700 BC when a tribe called Baluch migrated from Iran to the present Baluchistan (Bokhari 1975). The high frequency of IVSI-5 in Baluchis may be due to (a) founder effect (b) relative isolation (c) relatively small numbers and (d) few marriages outside the tribe preventing entry of new mutations.

Novel β-thalassaemia mutation:

Most β-thalassaemia mutations are well characterized and the chance of finding new mutation appears small. In this study only one out of 712 individuals (1240 β-thalassaemia chromosomes) investigated showed a new mutation. The 17bp deletion in Exon-III of β-globin gene was located between two copies of a CAG sequence present in Cd 125/126 and Cd 131. Sequence characteristics like a reiterated nucleotide sequence of two to eight base pairs (CAG in this case) separated by a few nucleotides appear to be involved in causation of nearly all small deletions (Bunn and Forget 1986).

Uncharacterized thalassaemia mutations:

In this study six subjects with apparent transfusion dependent thalassaemia were found to have only one mutation in the β-globin gene. All of these patients were heterozygous for a typical β-thalassaemia mutation, the second mutation could not be identified in the β-globin gene. In these patients there may be an unidentified mutation in another gene located elsewhere in the genome which is important in β-globin gene expression (Kazazian et al, 1990) or the mutation could be in the Locus Control Region (LCR) of the β-gene cluster (Cao et al, 1994). Co-inheritance of triplicate α-globin gene can also be a cause for transfusion dependency in these patients (Garewal et al, 1984). Lastly, these patients might have some other haematological abnormality e.g. congenital dyserythropoietic anaemia, pure red cell aplasia, or congenital sideroblastic anaemia which caused transfusion dependency and heterozygous β-thalassaemia could just be an incidental finding.

Screening strategy for prenatal diagnosis:

The objective of studying thalassaemia mutations is to carryout its prenatal diagnosis. Knowledge of the frequency of mutations in the target population is necessary for identifying the mutation(s) in at risk couples rapidly and efficiently. This study provides a comprehensive picture of the common, uncommon and rare β-thalassaemia mutations in the major ethnic groups of Pakistan. There is considerable heterogeneity within each ethnic group and the frequency of various mutations varies considerably between ethnic groups. However, in all groups up to five mutations account for over 80% of the alleles. Therefore the appropriate strategy is to first screen for the five commonest mutations in the ethnic group of the couple. The vast majority will be assigned a mutation at this stage. In the second round, if required, uncommon mutations may be sought and the rare mutations examined at the end.

The results of this study show that 79% of thalassaemia major patients had consanguineous parents and 88% of couples who were first cousins had identical mutations. This frequency declined to 58% for the more distantly related parents and to 43% for the unrelated parents. This can be very helpful for rapid identification of mutations in the consanguineous at risk couples.

ARMS is the ideal method for mutation analysis in a Pakistani setting. It is quick, efficient and there are minimum chances of a false positive or negative result provided necessary precautions are taken. DGGE can be used when an unknown mutation is encountered. In a very small proportion of couples who might have uncharacterized mutations Restriction Fragment Length Polymorphism (RFLP) can be used as a backup support for prenatal diagnosis (Varawalla et al, 1992).

Thalassaemia Intermedia:

Thalassaemia intermedia is a form of homozygous thalassaemia in which affected children can survive without transfusions or with only intermittent transfusions at least in the first few years of life (Lukens 1993). The main phenotypic determinant in a typical case of β-thalassaemia major is the degree of globin chain imbalance (Weatherall et al, 1989). Any factor that tends to ameliorate the chain imbalance would also influence the clinical outcome. At least three well

defined molecular mechanisms including mild β-thalassaemia mutations (Huisman 1990; Meloni et al, 1992), coincidence of α-thalassaemia (Wainscoat et al, 1983), and enhanced production of γ-globin chains (Thein et al, 1987) can lead to a thalassaemia intermedia phenotype. The modifying factors may be present independently or in combination in one patient (Ratip et al, 1997). Coincident α-thalassaemia reduces the chain imbalance by lowering the α-chain output, β^+-mutations act by minimally reducing the output of β-globin gene, and increased production of γ-chains results in improvement of the imbalance between α and non-α chains (Cao et al, 1994).

Diagnosis of thalassaemia intermedia is mostly retrospective. However knowledge about its molecular basis can help in prospective diagnosis at an early stage (Cao et al, 1994). The information about thalassaemia intermedia in subjects from the Indian subcontinent is scanty (Thein et al, 1988). This study initiates an analysis of the molecular basis of thalassaemia intermedia in Pakistani patients. The results indicate that the clinical outcome may be determined by the underlying molecular basis. The least severe clinical picture was associated with coincident α-thalassaemia ($-\alpha^{3.7}\alpha/-\alpha^{3.7}\alpha$). Coincidence of single gene deletion ($-\alpha^{3.7}\alpha/\alpha\alpha$) had no effect on the phenotype unless it was associated with a mild mutation (Gringras et al, 1994). β^+-mutations alone had minimal effect on the phenotype and Xmn-I +/+ genotype caused phenotypic effects that were in between the other two mechanisms. The results also suggest that the molecular basis of thalassaemia intermedia and therefore the clinical picture also vary considerably in the ethnic groups. In Punjabis, for example, Xmn-I +/+ genotype and in Pathans mild β-thalassaemia mutations like Cap+1 are important causes. In Sindhis, Baluchis and Mohajirs, coincident α-thalassaemia may be an important factor.

Studies in Sickle cell disease (SS) and β-thalassaemia heterozygotes have shown that A-T polymorphism at position -158 relative to $^{G}\gamma$ Cap site (recognized by Xmn-I) is associated with 3-11 fold increase in production per $^{G}\gamma$-globin gene (Gilman and Huisman 1985). The Xmn-I +/+ genotype acts by increasing the output of $^{G}\gamma$-globin gene in patients of homozygous β-thalassaemia. The beneficial effect of Xmn-I -/+ genotype, however, is not well defined. Winichagoon et al, (1993) have shown that -/+ genotype does not affect the phenotype. Whereas a study from Azerbaijan suggests that even Xmn-I -/+ genotype may alter the

phenotype (Dr. M. Petrou personal communication). The patients of homozygous β-thalassaemia with Xmn-I +/+ genotype usually have a thalassaemia intermedia phenotype because excessive γ-chains combine with free α-chains and results in increased production of Hb-F and lessening of dyserythropoiesis. The prevalence of Xmn-I polymorphism appears to vary between different populations. In the Mediterranean, for example, a +/+ geneotype is very uncommon (Gringras et al, 1994, Ratip et al, 1997). On the contrary it is quite frequent in the Asian Indians (Thein et al, 1987; present study) and the Southeast Asians (Winichagoon et al, 1993).

The study of association between Xmn-I "+" site and β-thalassaemia mutations is not large enough to draw firm conclusions. But the results give some indication that at least four mutations i.e. IVSI-5 (G-C), IVSI-1 (G-T), IVSII-1 (G-A) and Cd 30 (G-C) are more frequently associated with a "+" site than the other mutations. β-thalassaemia mutations are thought to have arisen in a relatively recent past on globin gene frameworks that are common in each population. The frequent finding of the same mutation linked to a "-" as well as a "+" site may be explained by recurrent mutations on different backgrounds, or multiple meiotic recombination events (Antonarakis et al, 1985). Multiple origin for IVSI-5 (G-C) is also supported by frequent finding of this mutation on several different globin gene haplotypes (Varawalla et al, 1992).

The frequency of β-thalassaemia mutations in patients of thalassaemia intermedia were different from thalassaemia major. In the former group milder mutations were over represented. There are also differences in the frequency of mutations between younger and older patients of thalassaemia major. Severe β-thalassaemia mutations are likely to be associated with more sever disease and therefore eliminated in the early years of life. Converse is true for the mild mutations. This highlights the difficulty in obtaining the true population frequencies of thalassaemia mutations. Representation form all age groups can minimize the selection bias due to age.

7 Prenatal diagnosis of thalassaemia

Introduction:

At present a regular service for prenatal diagnosis of thalassaemia does not exist in any Muslim country. Consequently, the response of a Muslim community to the availability of prenatal diagnosis is largely unknown. A service for prenatal diagnosis was introduced for the first time in Pakistan in May 1994. This chapter describes the process of introducing the service and the response of the affected families to the availability of this facility in Pakistan. The study period extended from May 1994 to July 1996.

Methods:

Initial preparation:

The service for prenatal diagnosis of thalassaemia was introduced after initial preparation of training and establishment of DNA laboratory facilities, training of an obstetrician in fetal sampling, and consultation with the religious scholars on termination of pregnancy for a genetic disorder. After initial preparation the facility was advertised for the at risk couples.

Training and establishment of laboratory facilities:

I was trained at the Perinatal Centre, Department of Obstetrics and Gynaecology, University College Hospital, London. The training involved analysis of DNA samples from thalassaemia patients collected in Pakistan. At the end of six months training from July to December 1993 information on the pattern of mutations in the ethnic groups of Pakistan was also available.

The necessary hardware and consumables for PCR based analysis of thalassaemia mutations were procured from the UK. On my return back to Pakistan, in January 1994, I established a DNA laboratory at the Department of Haematology, Armed Forces Institute

of Pathology (AFIP) Rawalpindi. The laboratory was completely functional by March 1994.

Training of obstetrician:

In April 1994 I came to know that Dr. Yasmeen Raashid, a female consultant obstetrician at Lady Wellington Hospital Lahore, had training in Chorionic Villus Sampling (CVS). Dr. Raashid had worked under the supervision of Dr. Gerald Mason, Fetomaternal Consultant, Leeds General Infirmary. She could do CVS by the transabdominal placental aspiration technique. Due to the availability of the obstetrician it became possible to offer prenatal diagnostic service. At the same time an experienced female obstetrician (Dr. Nadra Sultana) from the Military Hospital, Rawalpindi was also sent for training in CVS to the Department of Obstetrics and Gynaecology, University College Hospital, London. She had training for three months under the supervision of Prof. C. H. Rodeck and on her return to Pakistan in August 1994 she started doing CVSs at AFIP. Dr. Sultana was trained in using CVS biopsy forceps by the transabdominal route.

Consultation with religious scholars:

During the initial preparation I had personal communications with two of the renowned religious scholars regarding Islam's view point on termination of pregnancy for a serious genetic abnormality. A personal meeting with Dr. Malik Ghulam Murtaza was arranged at Lahore. Dr. Murtaza was of the opinion that termination of pregnancy is permissible for thalassaemia if carried out before 120 days of gestation. The other scholar, Mohammad Taqi Othmani, was approached through a letter. The reply by Mr. Othmani (Appendix-A) stated that Islam permits termination of pregnancy for a genetic disorder provided (1) the diagnosis is made by an honest person (2) severe nature of the disorder is confirmed (3) termination of pregnancy is carried out before 120 days of gestation. He clearly prohibited termination after 120 days even if the fetus is affected by a serious genetic abnormality. He considered termination after 120 days like killing an affected child.

Advertisement:

After establishing the laboratory facilities, completing arrangements for CVS and obtaining the opinion of religious scholars on termination of pregnancy, the service was

advertised. Most of the thalassaemics in Pakistan are treated at Fatimid Foundation thalassaemia centres, in Karachi, Multan, Lahore and Peshawar. Another NGO has its treatment centre in Rawalpindi. This provides an easy way of communicating with the affected families. The administration and the medical staff of the two charity organizations were informed about the availability of prenatal diagnosis. They in turn started communicating the information to the affected families. Instruction booklets written in "Urdu" the local language, were also provided for distribution to the affected families. The service was also advertised in the newspapers. A few programmes about the significance of prenatal diagnosis were also broadcasted on Pakistan Television Network.

Booking a couple for prenatal diagnosis:

The couples from Lahore and its surrounding areas reported at the obstetrics out patient clinic of Lady Wellington Hospital, Lahore and those from Rawalpindi and its surroundings reported at the Department of Haematology, Armed Forces Institute of Pathology, Rawalpindi. At the time of booking, the complete procedure of prenatal diagnosis its associated risks and the chances of error in diagnosis were fully explained. The chances of having an affected or unaffected child and the possibility of termination of pregnancy was also discussed. The ethnic group, parental consanguinity and the source from where the couple came to know about prenatal diagnosis was noted. The gestation of the pregnancy and the position of placenta were ascertained by ultrasound examination and the date of CVS was booked. Information about the socio-economic status, educational status, number of children, psychosocial burden and financial burden of thalassaemia was also recorded on a pre-designed questionnaire (Appendix-B). This was done with a view of identifying the type of couples who requested prenatal diagnosis.

Blood samples (3-5 ml) from the parents and an affected child, if available, were collected in EDTA containers. The couples examined at Lahore were mostly sampled at the time of CVS and the samples were despatched to AFIP for further analysis. In 7/158 (4.4%) couples the husband's sample could not be obtained because of their non-availability (5) or lack of interest (2).

Chorionic Villus Sampling:

Dr. Yasmeen Raashid did sampling at Lady Wellington Hospital, Lahore by a free hand transabdominal ultrasound guided aspiration technique (Brambati et al, 1988). Dr. Nadra Sultana did CVSs at AFIP, Rawalpindi by transabdominal biopsy forceps (Rodeck and Nicolini 1989). Most of the CVSs done at Lahore were collected in culture medium (RPMI-1640) and were transported to AFIP in ice within 24 hours. The samples were usually transported by the father or a close relative of the couple. When a person was not available to carry the samples or when the CVS was done before a closed holiday, a delay of up to 36 hours was encountered. Due to a short supply of RPMI-1640, ten CVSs performed at Lahore were collected and transported in normal saline. The samples obtained at AFIP were collected in normal saline and were processed without delay.

Mutation analysis of the parents:

Haematological diagnosis was available in only a few couples who requested prenatal diagnosis. Therefore prior to mutation analysis red cell indices were routinely done in all couples. Low MCV and MCH suggested thalassaemia trait whereas normal indices indicated either incorrect diagnosis or a silent β-thalassaemia mutation (Cap+1).

In the first round of mutation analysis the five most common mutations found in the ethnic group of the couple were tested. If the first round did not identify a mutation the second round of ARMS was done to screen for the uncommon mutations. The rare mutations were screened when the first two rounds failed to identify the mutation. In the last ten couples of the study, a multiplex ARMS PCR was also done. Three multiplex primer combinations were used to test each couple.

Where the father's DNA was not available, DNA from the affected child was used to characterize the father's mutation. The child's sample was tested in the usual way and when a mutation was found its normal allele was checked. The absence of a normal allele indicated that the father and the mother had the same mutation. If the affected child showed the normal allele the sample was further tested for other mutations.

Fetal diagnosis:

CVS is often contaminated with maternal decidua. Careful dissection under a microscope is essential to remove the maternal tissue (Cao and Rosatelli 1993). A stereo zoom microscope was used to clean the CVS. Branched and highly vascular villus structures were easily identified as compared to the more solid looking and much less vascular pieces of maternal decidua.

Mutation analysis:

Fetal diagnosis was accomplished by putting up ten ARMS reactions i.e. one for each parent, CVS in duplicate for the parent's mutation, one negative control for the parent's mutation, CVS in duplicate for the normal allele of the parent's mutation, negative and positive controls for the normal allele of the parent's mutations, and a reagent blank.

Linkage analysis:

When one or both mutations in the couple could not be identified by ARMS, diagnosis was done by linkage analysis. Six polymorphic sites closely linked to the β-globin gene were used as linkage markers.

Assessment of maternal contamination:

Maternal contamination was assessed in the first 10 CVSs by carrying out Apo-B VNTR analysis of the parents and the fetus (Rosatelli et al, 1992a). Although it would be preferable to do this on all samples to rule out maternal contamination as well as non-paternity, the cost must also be taken into account when applying this to a country such as Pakistan. Therefore in order to cut down the cost, this practice was discontinued and VNTR analysis was done only when difficulty was encountered in separating the maternal and the fetal tissues.

Follow-up:

The record of termination of pregnancy, complications of pregnancy, and postnatal out come following prenatal diagnosis was maintained in as many cases as was possible.

Response of the affected families to prenatal diagnosis:

At the end of the study, randomly selected 141 couples who visited Fatimid Thalassaemia Centre, Lahore for treatment of their children were interviewed to find out their response to the availability of prenatal diagnosis. The couples served as control for comparison with those who requested prenatal diagnosis. The response of the couples was recorded on a pre-designed questionnaire (Appendix-B) that included knowledge about the availability of prenatal diagnosis, any pregnancy after the availability of prenatal diagnosis, request for prenatal diagnosis in pregnancy (if any), request for prenatal diagnosis in future pregnancies and attitude towards termination of pregnancy.

Results:

Within 2-3 weeks from the announcement of the service the first couple came forward with a request for prenatal diagnosis. Several other couples who were avoiding a pregnancy came for counselling and information about the test. Thereafter the couples appeared at regular intervals and a steady increase in the number of requests for prenatal diagnosis was observed. During the two years of the study a total of 158 couples requested prenatal diagnosis. This included 118 (74.7%) Punjabi, 30 (19%) Pathan, 4 (2.5%) Baluchi, 4 (2.5%) Mohajir and 2 (1.3%) Sindhi couples. Fig: 7.1 shows the number of couples who requested prenatal diagnosis in each quarter of the year. The distribution of the couples according to the place of origin is presented in Fig: 7.2.

Almost all of the couples were informed about prenatal diagnosis by their doctors. Table: 7.1 gives a summary of the medium of information for the couples who requested prenatal diagnosis. A significant proportion (21%) was inspired by the experience of other couples who already had used prenatal diagnosis and many of whom had normal children due to the test. In 31% of the couples one member, usually the mother, had watched a programme on television. The information booklets had either not been delivered to all couples or many were not able to read because of illiteracy. Only two couples came with a request for prenatal diagnosis after having read the information booklet alone.

Table: 7.1. The medium of information for the couples who requested prenatal diagnosis.

Informed by:	Number:	Percent:
Treating doctor	153/158	96.8%
Television	45	28.5%
Other parents who had used prenatal diagnosis	33	20.9%
Information booklet	2	1.3%

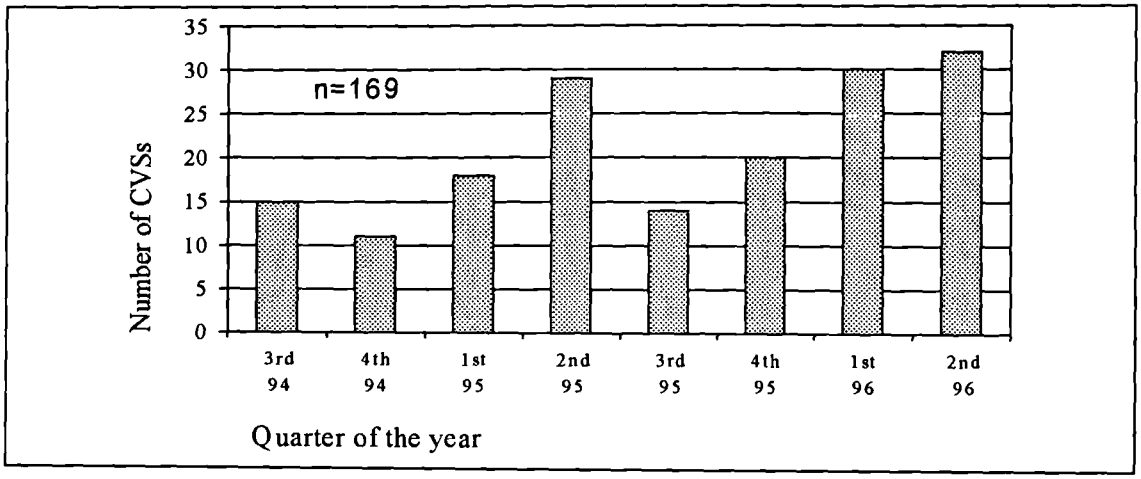


Fig: 7.1. The number of CVSs done for prenatal diagnosis of thalassaemia in the two study years.

Characteristics of the couples who requested prenatal diagnosis:

The characteristics of couples who requested prenatal diagnosis (cases) were compared with the randomly interviewed couples (controls) and the comparison was as follows:

Socio-economic status:

There were significantly more couples in the high socio-economic group (Table: 7.2) who requested prenatal diagnosis (p=0.0126).

Educational standard:

The educational standard of the couples who requested prenatal diagnosis was better as compared to the controls (Table: 7.3).

Fig: 7.2. Area wise distribution of 158 couples who requested prenatal diagnosis. Most of the couples came from areas in and around Lahore and Rawalpindi.

Number of children:

Only 2/158 (1.3%) couples who requested prenatal diagnosis did not have an affected child before the test. In one couple the father was a laboratory technician and therefore was well informed of thalassaemia. In the other couple the mother was a doctor who had an affected first cousin. Comparison between the two groups of couples (Fig: 7.3) showed a near normal distribution of the number of children in the controls. Whereas the distribution was markedly skewed towards the left in the couples who requested prenatal diagnosis. This was mainly due to the younger age group of the couples who requested prenatal diagnosis.

Effect of thalassaemia on family life:

The family life of 69% of the couples who requested prenatal diagnosis was affected a lot due to thalassaemia as compared to 83% in the controls (Table: 7.4).

Financial burden of thalassaemia:

There was a significant differences in the number of couples in the high income group between the cases and the controls (Table: 7.5). Financial burden of thalassaemia was also significantly different (Table: 7.6). Severe burden was felt by 58% of the couples who requested prenatal diagnosis as compared to 32% in the control group (p=<0.001).

Table: 7.2. Comparison of the socio-economic status of the group of couples who requested prenatal diagnosis and the controls.

Socio-economic status:	**Low:**	**Middle:**	**High:**
Cases (n=158)	99 (62.7%)	38 (24.1%)	21 (13.3%)
Controls (n=141)	110 (78.0%)	25 (17.7%)	6 (4.3%)
p:	0.224	0.279	0.0126

Table: 7.3. Comparison between the educational standard of the group of couples who requested prenatal diagnosis and the controls.

Education:	**None**	**Primary**	**Matric***	**Above matric**
Father's education:				
Cases (n=158)	12 (7.6%)	32 (20.3%)	43 (27.2%)	71 (44.9%)
Controls (n=141)	35 (24.8%)	29 (20.6%)	39 (27.7%)	38 (27.0%)
p:	0.0005	0.956	0.948	0.0267
Mother's education:				
Cases (n=158)	36 (22.8%)	54 (34.2%)	36 (22.8%)	32 (20.6%)
Controls (n=141)	61(43.3%)	45 (31.9%)	19 (13.5%)	16 (11.3%)
p:	0.0071	0.768	0.084	0.074

*matric is an educational standard that is equivalent to O-levels.

Table: 7.4. Comparison of the effect of thalassaemia on the family life of couples who requested prenatal diagnosis and the controls.

Effect on family life:	**A little:**	**Often:**	**A lot:**
Cases (n=158)	10 (6.3%)	39 (24.7%)	109 (69.0%)
Controls (n=141)	2 (1.4%)	22 (15.6%)	117 (82.9%)
p:	0.038	0.112	0.295

Table: 7.5. Comparison of the monthly income of the couples who requested prenatal diagnosis and the controls.

Monthly income (Rs):	**< 5,000:**	**5,000-10,000:**	**> 10,000:**
Cases (n=158)	99 (62.6%)	38 (24.1%)	21 (13.3%)
Controls (n=141)	108 (76.6%)	27 (19.1%)	6 (4.3%)
p:	0.267	0.410	0.0126

Table: 7.6. Comparison of the effect of thalassaemia on the financial situation of the couples who requested prenatal diagnosis and the controls.

Effect on financial Situation:	**A little:**	**Often:**	**A lot:**
Cases (n=158)	19 (12.0%)	47 (29.7%)	92 (58.2%)
Controls (n=141)	48 (34.0%)	54 (38.3%)	39 (27.7%)
p:	<0.001	0.272	<0.001

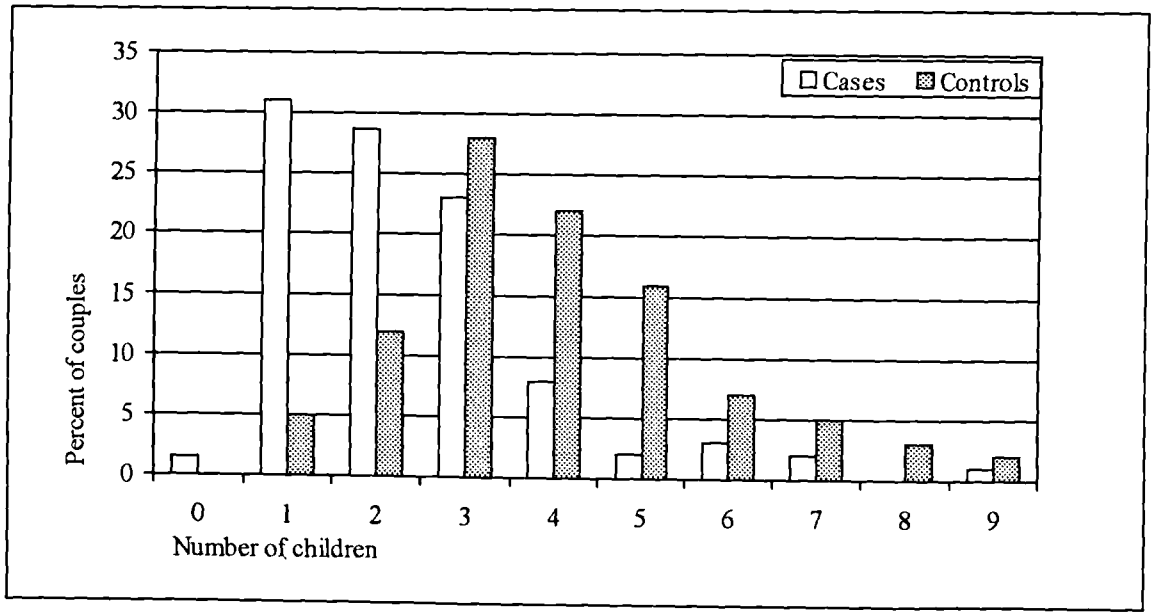


Fig: 7.3. Comparison of the number of children in the couples who requested prenatal diagnosis and the control group. The distribution in the control group is near normal whereas in the couples who requested prenatal diagnosis the distribution is markedly skewed towards the left. The data is presented as percent of the actual number of couples in each group.

Fetal diagnosis:

Chorionic Villus Sampling:

Out of 158 couples who requested prenatal diagnosis 9 (5.7%) used it twice and one couple used it three times. Seven couples who requested prenatal diagnosis more than once, had a previous diagnosis of an affected fetus. A total of 169 CVSs were done that included 148 for the couples who requested the test once, 18 for the couples who requested it twice and 3 for a couple who requested it thrice. There were 161 single pregnancies and four twin pregnancies. Of 169 CVSs 89 (53%) were done at Lahore and the remaining 80 (47%) were done at AFIP, Rawalpindi. Most of the CVSs were done between 10 and 16 weeks of gestation (Fig: 7.4). Only 11/169 (6.5%) were done after 16 weeks and two were done after 18 weeks. In 8 cases CVS was repeated because either the first attempt was not successful or the sample obtained was inadequate. Five of the later CVSs were done at AFIP by a CVS biopsy forceps, while the other three were done at Lahore where the placental aspiration technique was used.

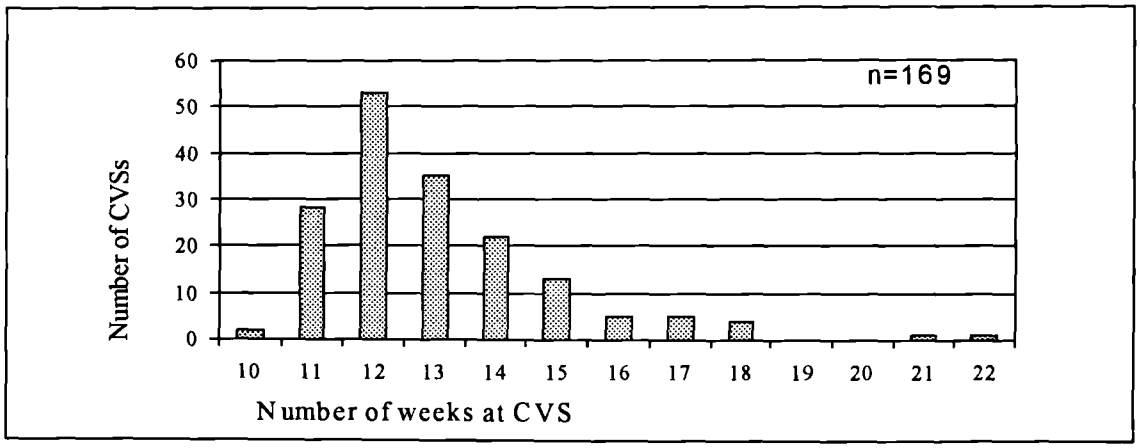


Fig: 7.4. The number of weeks of gestation in 169 CVSs carried out at Lahore and Rawalpindi.

Mutation analysis in the parents:

Measurement of red cell indices prior to mutation analysis was found to be useful. In one couple it indicated an incorrect diagnosis of thalassaemia. This was later confirmed by Hb-A_2 estimation. Bone marrow aspiration of the affected child showed the features of congenital dyserythropoietic anaemia. The couple was excluded from the study as CVS had not yet been carried out. In nine couples one of the parents had either completely

normal or borderline red cell indices. Five of these had Cap+1 mutation and the remaining four, who had borderline red cell indices had a severe β-thalassaemia mutation.

Standard ARMS PCR:

The five common mutations accounted for 257/316 (81.3%) of the alleles (Table: 7.7). After the first round of PCR both members were assigned a mutation in 115/158 (72.8%) couples. In the second round of PCR another 54 (17%) of the alleles were identified and 153/158 (96.8%) of the couples were assigned mutations. After the final round of PCR 313/316 (99.1%) of the alleles were identified and 155/158 (98.1%) couples were completely assigned their mutations. In two of the remaining three couples only one parent and in the third couple both parent's mutations could not be characterized. In the first couple sequencing done at a later stage showed -88 (C-T) mutation and in the second couple sequencing of the entire β-gene failed to reveal a mutation. The sample from the third couple awaits sequencing.

Multiplex ARMS PCR:

The multiplex ARMS PCR proved to be technically feasible for mutation detection in all of the couples tested (Fig: 7.5). The results of multiplex and standard ARMS PCR were in complete agreement. However, the problem of false positive results with the primers described in Chapter: 3 became more pronounced because of the sensitivity of silver staining method. This was overcome by taking precautions already described and by loading only 1-2 μl of the amplified product on the gel. The method was quick and the addition of many different primers, that can be a potential source of error if addition of a primer is omitted were simplified. The polyacrylamide gels as compared to the agarose gels were more difficult to prepare and handle. The main advantage was the use of only six reactions per couple that could screen over 97% of the mutations. This saved a substantial amount of time and money.

Analysis of mutations in the fetal DNA:

While testing the fetal DNA parental samples were included to reconfirm the findings of initial screening. This was essential to prevent clerical mistakes. Negative and positive

controls were also included to safeguard against technical errors. The presence of the parental mutation and the absence of its normal allele in the test sample indicated a homozygous fetus (Fig: 7.6a). The presence of mutant and the normal alleles indicated a heterozygous fetus, a normal allele but no mutation indicated a normal fetus. In cases where both the parents had different mutations and both were also present in the fetal sample, a diagnosis of an affected fetus was made. Similarly, the presence of one of the mutations indicated a heterozygous fetus.

Linkage analysis:

In three couples where one or both of the mutations remained uncharacterized after screening with the ARMS had linkage analysis done. In two couples one mutation was known and the same mutation was also present in the fetal DNA. Linkage analysis in these couples was done as even partially informative markers were also helpful. In the third couple both mutations remained uncharacterized,, for diagnosis a polymorphism at 5'ψβ was informative and antenatal diagnosis was successfully accomplished (Fig: 7.6b).

Results of fetal diagnosis:

In the 169 fetuses (CVSs) diagnosed, 43 (25.4%) were affected (homozygous/compound heterozygous), 44 (26.0%) were normal, and 82 (48.5%) were heterozygous for thalassaemia.

Maternal contamination:

VNTR analysis in the first ten CVSs ruled out maternal contamination. Experience in the first ten CVSs showed that meticulous cleaning of the CVS is enough to safeguard against maternal contamination.

Time frame of prenatal diagnosis:

The usual time taken for a prenatal diagnosis varied between one day to one week. In couples where mutation analysis had previously been done, the CVS results were usually available the following day. The results were considerably delayed where CVS was done on the same day as blood was collected. Linkage based diagnosis took up to one week.

Table: 7.7. Characterization of β-thalassaemia mutations by ARMS in 158 couples who requested prenatal diagnosis.

ARMS PCR:		**Results:**	
Round:	**Mutations screened:**	**No of alleles detected:**	**No of couples resolved:**
1st	Fr 8-9 (+G) IVSI-5 (G-C) Fr 41-42 (-TTCT) IVSI-1 (G-T) Del 619 bp	257/316 (81.3%)	115/158 (72.8%)
2nd	Cd 5 (-CT) Cd 30 (G-C) Cd 30 (G-A) Cd 15 (G-A) Fr 16 (-C) Cap +1 (A-C) IVSII-1 (G-A)	54/316 (17.1%)	153/158 (96.8%)
3rd	Hb-E IVSI minus 25 (-25bp) Fr 47-48 (+ATCT) IVSI-1 (G-A) Cd 39 (C-T)	2 (0.6%)	155/158 (98.1%)
-	Uncharacterized mutations:	3 (0.9%)	-
Total:		313/316 (99.1%)	155/158 (98.1%)

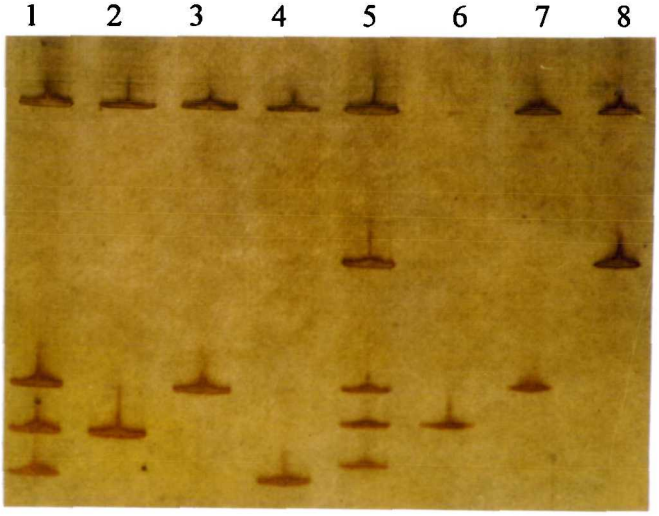


Fig: 7.5. Silver stained polyacrylamide gel electrophoresis of multiplex ARMS PCR for β-thalassaemia mutations. Lane-1 and 5 show allelic ladders for AD-2 and AD-1 respectively. Lane 2, 3, and 4 show AD-2 multiplex reactions positive for Fr 16, Cd 30, and Cd 5 respectively. Lanes 6, 7, and 8 show AD-1 multiplex reactions positive for del 619, IVSI-5, and Fr 41-42 respectively. Control bands of 861 bp are visible in all lanes except lane 6 where it indicates homozygous del 619.

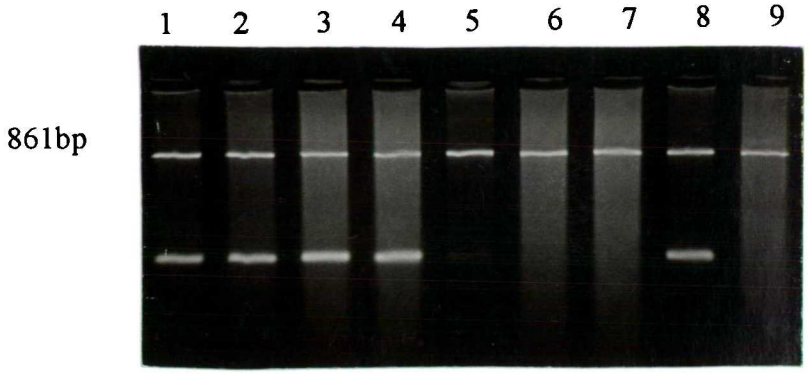


Fig: 7.6a. Ethidium bromide stained agarose gel electrophoresis of a standard ARMS PCR for prenatal diagnosis of thalassaemia. All lanes show 861bp control bands. Lanes 1-4 show 285 bp bands of IVSI-5. Lane 1 and 2 contain parent's samples while lane 3-4 contain fetal DNA in duplicate. Lane 5 is a negative control for IVSI-5. Lanes 6-7 show absence of bands for the normal allele of IVSI-5 in the fetal DNA. Lane 8 and 9 are positive and negative controls for the normal allele of IVSI-5. The results indicate a homozygous fetus.

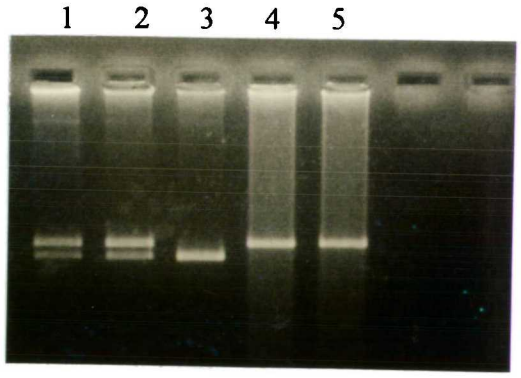


Fig: 7.6b. Prenatal diagnosis by linkage analysis at the 5'ψβ polymorphism. Figure shows the Hinc-II digest of the PCR amplified fragments after electrophoresis on agarose gel. Lane 1: father (-/+), Lane 2: mother (-/+), lane 3: affected child (+/+) and lane 4 & 5: CVS (-/-). The findings suggest that thalassaemia mutation is linked to "+" site and the CVS is "Normal".

Follow-up:

Complete follow-up was possible in 117/158 couples the rest were lost to follow-up.

Complications of CVS:

In 78 pregnancies that were not terminated and were available for follow-up, 7 (8.9%) spontaneously terminated within 2 weeks following a CVS. The complications occurred in 3/37 (8.1%) and 4/41 (9.7%) at the centres in Lahore and Rawalpindi respectively. The miscarriages at Lahore occurred during the first 20 CVSs whereas at Rawalpindi these were evenly spread over the entire study period.

Termination of pregnancy:

Out of 43 women diagnosed with an affected fetus, 42 were available for follow-up and all but three (93%) terminated their pregnancy. In 32/39 (82%) cases termination was done before 15 weeks of gestation (Fig: 7.7). In three cases termination was done at or after 17 weeks (17, 18 and 21 weeks). Three couples did not terminate pregnancy due to religious reasons. In all three the CVS was carried out between 13 and 14 weeks of gestation and the results were conveyed within one week of the test. These couples felt that the pregnancy was far too advanced to terminate. In a twin pregnancy one fetus was affected and the other was normal, as selective termination could not be carried out, the parents requested termination of pregnancy. It was not possible to access most of the terminated fetuses, but in two fetuses referred for DNA analysis the diagnosis of homozygous thalassaemia was reconfirmed.

Post natal outcome:

Sixty two children born after prenatal diagnosis of "normal" or "trait" were available for follow-up. Twenty five children were brought to AFIP for reconfirmation of prenatal diagnosis. In the remaining children reconfirmation of diagnosis was not possible as the couples had come from far off places. Prenatal diagnosis was reconfirmed in 25 children by Hb-A_2 or DNA analysis. In 3/37 couples that were contacted by letter informed that the child was tested at a local laboratory and the prenatal diagnosis was correct. The remaining 34 children were found to be apparently healthy and none required blood

transfusions. The laboratory reconfirmation of diagnosis in these children was not done. Since the introduction of prenatal diagnosis no misdiagnosis has been reported. None of the children born after CVS had any apparent congenital malformation. Three couples with a fetal diagnosis of thalassaemia major refused termination of pregnancy. One of the couples was lost to follow up. The other two children have transfusion dependent thalassaemia major.

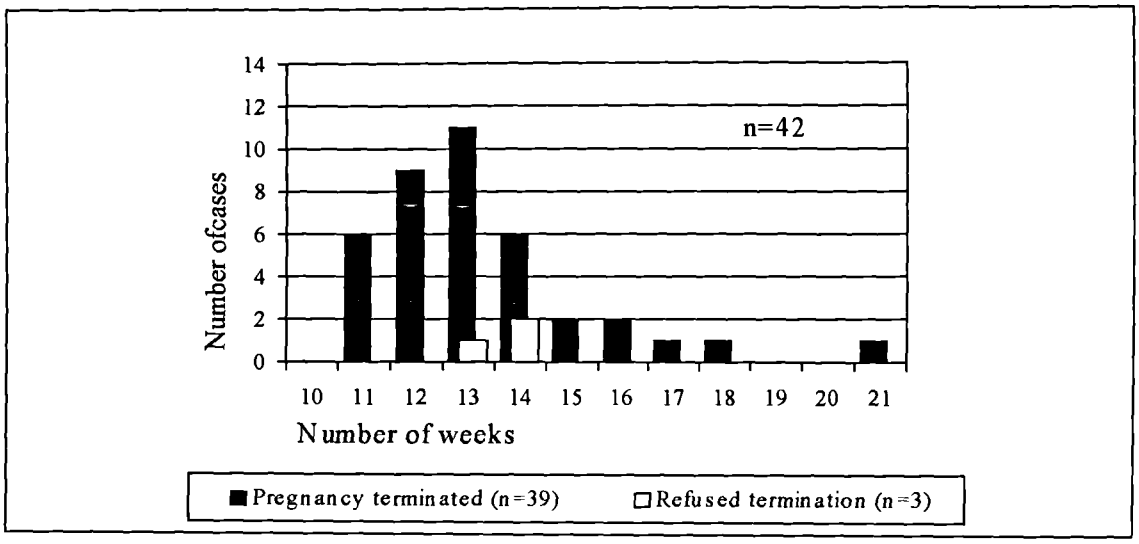


Fig: 7.7. The response of the couples to termination of pregnancy after fetal diagnosis of thalassaemia major.

Response of the families to prenatal diagnosis:

Knowledge about the availability of prenatal diagnosis:

Out of 141 randomly interviewed couples, 101 (72%) knew the test was available in Pakistan. Three couples had no interest in prenatal diagnosis because they had already completed their families. The remaining 38 (27%) were completely ignorant about the test facility.

Attitude towards prenatal diagnosis:

During the period when prenatal diagnosis was available 62 couples had a pregnancy. Two women (3.1%) aborted spontaneously prior to prenatal diagnosis. Twenty five of the remaining 60 couples (41.6%) were either not aware of the test or came to know about it very late and therefore did not request prenatal diagnosis. There were 3/60 (5%) couples

who were not aware of their risk because they did not have an affected child. In 32 couples who had a pregnancy and were aware of the test facility, 16 (50%) requested it and another two were waiting to use the test (Table: 7.8). Two couples preferred to terminate the pregnancy rather than use prenatal diagnosis. Twelve out of 32 (37.5%) couples knew about prenatal diagnosis at the time of pregnancy but did not use it due to reasons given in Table: 7.9. Six couples had no clear explanation for refusing prenatal diagnosis, two couples avoided the test due to cost and in another two the mothers were afraid of the test. There was a serious disagreement between the parents in two couples i.e. one favoured the test and the other did not.

Response of the couples to the possibility of prenatal diagnosis in a future pregnancy showed that 29 (20.6%) had completed their families. In the remaining 112 couples, 102 (91%) were in favour of prenatal diagnosis, and six (5.4%) were not sure. Only 4/112 couples (3.6%) were not in favour of prenatal diagnosis. Of the 102 couples who favoured prenatal diagnosis, 75 (73.5%) would request it unconditionally, but 27 (26.5%) would request it if it were free of cost. Most of the later 27 couples (93%) were from the low income group.

Attitude towards termination of pregnancy:

When questioned, 124/141 (87.4%) couples felt they would terminate an affected fetus. Only 2% had a negative attitude towards termination of pregnancy (Fig: 7.8).

Table: 7.8. Response to prenatal diagnosis of 32 couples who had a pregnancy and were also aware of the test facility.

Response:	**Number (%):**
Requested prenatal diagnosis	16/32 (50%)
Preparing to request prenatal diagnosis	2/32 (6.3%)
Did not request prenatal diagnosis	12/32 (37.5%)
Terminated pregnancy prior to prenatal diagnosis	2/32 (6.3%)
Total	32 (100%)

Table: 7.9. Reasons for not requesting prenatal diagnosis in 12 couples who had a pregnancy and also knew that the test was available.

Reasons:	Number (%):
Unclear reasons	6/12 (50%)
Disagreement between the parents	2/12
Fear of undergoing the test	2/12
High cost of the test	2/12
Total	12 (100%)

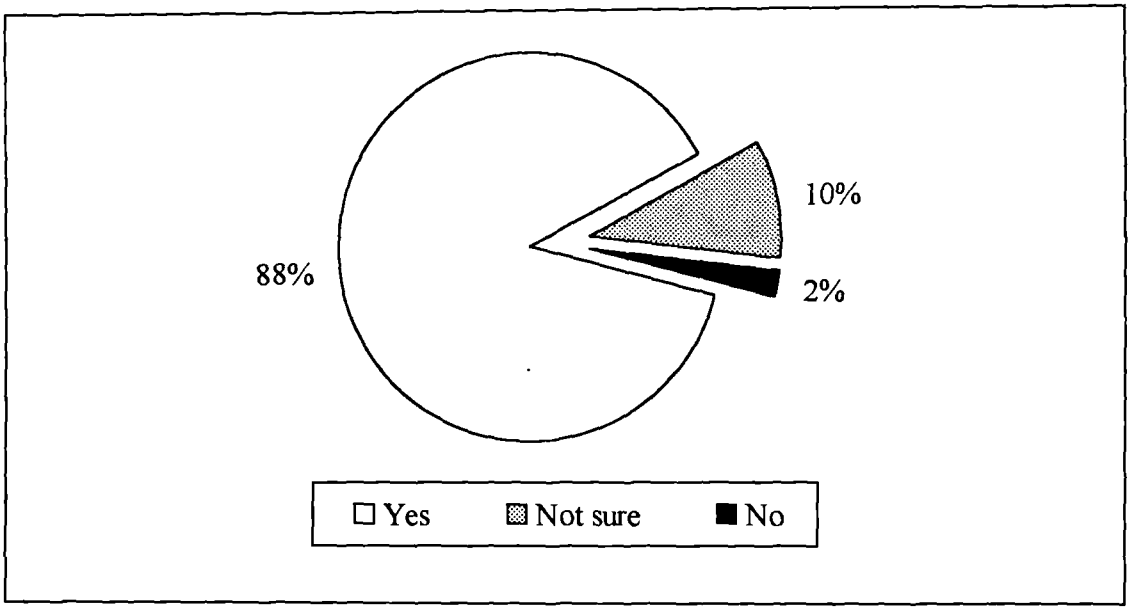


Fig: 7.8. Attitude of the randomly interviewed thalassaemic couples towards termination of pregnancy.

Discussion:

Technical feasibility:

The first step in fetal diagnosis is the analysis of parent's mutations. In this study direct mutation analysis was possible in over 98% of the couples. This makes it the method of choice for fetal diagnosis in most cases. The procedure is quick and reliable and is also cost effective. The multiplex ARMS can further reduce the time required in mutation analysis from two days to just one day. Similarly, the cost of mutation analysis per person can also be reduced considerably (Rs. 500 ($12.5) instead of Rs. 800 ($20); Chapter: 8).

It is important to ascertain the correct diagnosis of thalassaemia before mutation analysis is done. Thalassaemia is the commonest cause of transfusion dependent anaemia in Pakistan. Experience at AFIP shows that congenital dyserythropoietic anaemia (CDA), an autosomal recessive disorder, is the next most common cause of transfusion dependency (unpublished observation). Both the disorders have similar clinical picture. Therefore it is likely that a small proportion of patients having transfusions in Pakistan may actually be suffering from CDA. It may be useful to screen the couples who request prenatal diagnosis by red cell indices as it can help in differentiating between thalassaemia and other causes of transfusion dependency.

Chorionic villus sampling is the best approach for fetal sampling because it can be done in the first trimester of pregnancy and the associated risk of miscarriage is also less than 1% (Centre for Disease Control and Prevention, Atlanta 1995). The results of this study indicate that over 90% of Pakistani couples would request early prenatal diagnosis. Therefore CVS is the best option for fetal sampling. Procedure related complications in this study were high (9%). However in the early part of a new programme high miscarriage rates are not unusual (Old et al, 1986). The use of rigid biopsy forceps is more traumatic and may be associated with more frequent complications than aspiration needles (Golbus and Appelman 1990). The rate of complications is also related to the operator skill. CVS should be done at a centre where good ultrasound support is available. The samples can be easily transported to a central DNA laboratory either in RPMI-1640 or even normal saline.

CVS is often contaminated by maternal decidua and its meticulous cleaning is essential (Cao and Rosatelli 1993). VNTR analysis can rule out maternal contamination (Rosatelli et al, 1992a). This also safeguards against errors due to non-paternity. In this study financial constraints precluded VNTR analysis in most of the cases and meticulous cleaning of the CVS had to be accepted as adequate in the local circumstances.

In this study fetal diagnosis was possible by direct mutation analysis in over 98% of the couples. In the remaining couples it was possible to use RFLP as backup support. Inclusion of appropriate controls and reagent blanks (Kwok and Higuchi 1989) increased

the confidence in accuracy of diagnosis. The follow-up in 62 children born after a prenatal diagnosis indicates that the method is accurate and reliable for large-scale application.

Response of the couples to prenatal diagnosis:

Almost all of the couples in this study were retrospectively identified and they had at least one affected child. Their response to the availability of the test and to termination of pregnancy was very good. The couples who requested prenatal diagnosis mostly had small families, better education and higher socio-economic status. The response of the randomly interviewed at risk couples showed that the majority (72%) knew about the test and over half of the couples also had a positive attitude towards it. Careless attitude and the cost of the test were seen as the main reasons for avoiding the test. Sustained efforts at counselling the at risk couples can substantially improve the attitude of the couples. Subsidizing the cost of the test can also improve the acceptance rate for prenatal diagnosis especially amongst the low socio-economic group of couples.

Prenatal diagnosis and termination of pregnancy:

The community would accept the concept of prenatal diagnosis if it is compatible with the religious and cultural beliefs. Two renowned religious scholars in Pakistan, when asked to give opinion on permissibility of prenatal diagnosis and termination of pregnancy for thalassaemia gave a verdict in its favour provided the termination is carried out before 120 days (17 weeks) of gestation. The couples who were reluctant to use prenatal diagnosis were greatly relieved to learn that terminating a pregnancy is permissible under special circumstances. Most of the couples who requested prenatal diagnosis in this study had little hesitation in terminating an affected pregnancy. Only 3/42 (7%) couples refused termination and all three did so on religious grounds. A high rate of acceptance of termination may be because only a selected group of couples, who were prepared to accept termination of pregnancy, requested the test. However most of the prospectively interviewed couples (87% in this study) would terminate an affected fetus.

8 Cost and benefit of thalassaemia prevention

Introduction:

An important element in programme development is analysis of the cost and benefit of different approaches. There is good evidence that implementing policies for prevention of genetic disorders leads to significant financial savings and that these are particularly marked in case of thalassaemia (Cao 1987). Alwan and Modell (1997) have worked out the minimum cost of treatment per patient per year and the cost of prevention of thalassaemia in the Eastern Mediterranean Region. However, as far as Pakistan is concerned these estimates are a theoretical exercise. The aim of this chapter is to illustrate the cost of treatment and compare it with the cost of prevention and to show how such analysis can contribute towards the development of appropriate policies in Pakistan.

Methods:

In order to analyze the cost effectiveness of thalassaemia prevention the following parameters were evaluated: (1) estimated number of new births of thalassaemia major per year (N), (2) cost of treatment (3) cost of carrier screening and (4) cost of prenatal diagnosis. The costs were calculated in Pak Rupees. Equivalent cost in US dollars, at the rate of Rs. 40.00 per $ was also calculated.

Results:

Estimated number of thalassaemia major births per year (N):

Calculation of the annual number of thalassaemia major patients in Pakistan are discussed in Chapter: 4. The annual birth rate of thalassaemia major is estimated at approximately 4550. In addition births of 550 patients of a clinically significant abnormal haemoglobin with or without thalassaemia are also expected. This number also includes approximately 200 patients of Hb-S

and Hb-E homozygotes who do not normally require blood transfusions. The total number of patients (N), who would require transfusions, is estimated at 4900. However, for the sake of calculations a round figure of 5000 new cases per year is used.

Cost of treatment:

Each year a patient of thalassaemia major on an average requires 26 units of blood and approximately 364 grams of desferal (Table: 8.1). The cost of one bag of blood (Table: 8.2) is calculated at Rs. 1540 and the average annual cost on blood transfusions alone is estimated at Rs. 40,000. The cost of desferal therapy per year is estimated at Rs. 116,000 (Table: 8.1). The annual cost of investigations per patient (Table: 8.3) is estimated at Rs. 2000. The total cost of management per patient per year (Table: 8.4) is estimated at Rs. 160,000 ($ 4000).

Cost of screening:

The cost of screening for β-thalassaemia includes the cost of sample collection tube, disposable syringe, testing the sample on red cell analyzer, Hb-A_2 estimation by cellulose acetate electrophoresis, multiplex ARMS PCR and the labour. In Chapter: 5 it was shown that all cases do not require every investigation and the number of individuals requiring Hb-A_2 estimation or DNA analysis are also different in the two groups of people studied i.e. the pregnant women and the index families. Therefore the cost per individual would also be different in these groups. The cost of screening 1000 pregnant women (Table: 8.5) is calculated at Rs. 60,000 ($ 1500) and that of screening 1000 individuals from thalassaemic families is Rs. 78,000 ($ 1950).

Table: 8.6 compares the cost of antenatal versus family screening in terms of cost per person screened, cost per carrier detected and cost per at risk couple detected. In the antenatal set up 50 carriers per 1000 (5%) and in the families 300 carriers per 1000 (30%) are expected. The expected number of at risk couples in the antenatal set up would be 2.5/1000 (0.25%) whereas in the family screening it would be 6/1000 (Chapter 5 & 9). The cost per carrier as well as per at risk couple detected is markedly different between the two groups.

Table: 8.1. Basic annual requirement for treating one patient with thalassaemia major, related to age. The figures for donor blood consumption are adopted from Alwan and Modell (1997). An average dose of desferal (35 mg/kg/day) has been used for all calculations (Cao et al, 1992).

Age group (years)	Mean weight (kg)	Units of donor blood/year *	Desferal @35 mg/kg/day X 5 days a week.	Cost @ Rs. 320 per gram.
1-5	15	10	130 g	42,000
6-10	25	17	230 g	74,000
11-15	40	27	390 g	125,000
16-21	55	37	520 g	167,000
21-25	60	40	550 g	176,000
All ages:	39	26	364 g	116,000 ($ 2900)

* at about 300 ml/kg/years: 1 unit = 450ml donor blood

Table: 8.2. An estimate of the expenditure on preparation of one bag of blood. The prices are based on calculation by the Armed Forces Institute of Pathology, and the Armed Forces Institute of Transfusion, Rawalpindi, Pakistan.

• Blood bag and transfusion set:	Rs. 80.00
• Screening for Hepatitis B:	Rs. 150.00
• Screening for Hepatitis C:	Rs. 600.00
• Screening for HIV:	Rs. 400.00
• Blood grouping:	Rs. 60.00
• Cross match:	Rs: 250.00
• Total cost:	Rs: 1540.00 ($ 38.00)

Table: 8.3. Estimated cost of investigations per patient of thalassaemia major per year. The investigations include those recommended by Cao et al (1992) and the prices are based on those calculation by the Armed Forces Institute of Pathology, Rawalpindi, Pakistan.

• Haemoglobin estimation (X 24):	Rs. 480.00
• Liver function tests (X 2):	Rs. 570.00
• Serum ferritin (X 2):	Rs: 350.00
• Miscellaneous investigations:*	Rs. 600.00
• Total:	Rs. 2000.00 ($ 50)

* Miscellaneous investigations: thyroid function tests, growth hormone, blood sugar, ECG and Muga-I scan of heart etc.

Table: 8.4. Total cost of treatment for thalassaemia per patient per year.

Blood transfusions X 26 units per year (Table: 8.1 & 8.2):	Rs. 40,000 ($ 1000)
Desferal (Table: 8.1):	Rs. 116,000 ($ 2900)
Investigations (Table: 8.3):	Rs. 2000 ($ 50)
Miscellaneous expenditures:*	Rs. 2000 ($ 50)
Total cost:	Rs. 160,000 ($ 4000)

* Miscellaneous expenditures include cost of labour, antibiotics, occasional hospital admissions, and splenectomy if required.

Table: 8.5. Estimated cost of screening for β-thalassaemia in pregnant women and index families.

Investigations consumable items and manpower:	**Cost X 1: (Rs)**	**Cost per 1000 individuals screened (Rs):**			
		Antenatal screening:		**Family screening:**	
		Requirement:	**Cost:**	**Requirement:**	**Cost:**
Sample tube	5	100%	5,000	100%	5,000
Disposable syringe	3	100%	3,000	100%	3,000
Haematology	20	100%	20,000	100%	20,000
Hb-A_2 estimation	35	20%	6,650	50%	17,500
DNA analysis	350	3.5%	12,250	5%	17,500
Technicians	3000 pm	X 2	6,000	X 2	6,000
Phlebotomist	2000 pm	X 1	2,000	X 2	4,000
Doctor:	5000 pm	X 1	5,000	X 1	5,000
Total	-	-	59,900 ($ 1500)	-	78,000 ($ 1950)

pm: per month salary

Table: 8.6. Comparison of the cost of antenatal versus family screening in terms of cost per person screened, cost per carrier detected and cost per at risk couple detected.

Cost of screening (Rs.):		
	Antenatal:	**Index families:**
Per person:	60	78
Per carrier detected:	1200	260
Per at risk couple detected:	24000	13000

Cost of prenatal diagnosis:

Chorionic villus sampling (CVS):

All of the CVS in this study were done free of cost. It is therefore difficult to calculate the cost of each CVS. The cost of a CVS aspiration needle is Rs. 4000 and the cost of a biopsy forceps is Rs. 22000. However, the needles as well as the forceps can be reused. Therefore the cost of a CVS is equivalent to the salary of the obstetrician and the ultrasound operator (approximately Rs. 20,000 per month). If approximately 100 CVSs are done every month then the cost per procedure will be Rs. 200.

Laboratory diagnosis:

The cost of mutation analysis (Table: 8.7) by the standard ARMS PCR per person is calculated at Rs. 800 ($ 20) and by multiplex PCR is Rs. 500 ($ 12.5).

Cost of termination of pregnancy:

Cost of termination involves the cost of the procedure and the cost of hospital admission for 2-3 days. The total cost is calculated at approximately Rs. 4000 ($ 100). Since only about 25% of women who request prenatal diagnosis would require termination, the actual cost per diagnosis will be Rs. 1000 ($ 25).

Total cost:

The total cost of one prenatal diagnosis (Table: 8.8), including the cost of CVS, mutation analysis, and termination of pregnancy when required, is estimated at Rs. 3600 ($ 90) by standard ARMS PCR and Rs. 3000 ($ 75) by multiplex ARMS PCR.

Discussion:

The success of a policy depends on whether genetic counselling is provided retrospectively i.e. after the birth of an affected child or prospectively i.e. to at risk individuals and couples without an affected child (Alwan and Modell 1997). The potential effect of a policy also depends on its acceptability to the families concerned and the society as a whole.

The concept of thalassaemia prevention is new to Pakistan. This pilot study clearly shows that carrier screening as well as prenatal diagnosis are technically feasible and also accepted by the majority of affected families. Consequently, formulation of policies incorporating prenatal diagnosis is feasible for a large-scale thalassaemia prevention programme in Pakistan. In the subsequent sections the cost and benefit of three basic policies, including no prevention, retrospective counselling with prenatal diagnosis and prospective identification of at risk couples and the offer of prenatal diagnosis, will be discussed. For the purpose of discussion a hypothetical situation is assumed in which the total number of thalassaemics is equal to the number of new births of thalassaemia in one year.

Table: 8.7. Estimated cost of mutation analysis for β-thalassaemia per person.

Item:	**Cost per person (Rs.):**	
	Standard ARMS PCR:	**Multiplex ARMS PCR:**
Sample tube and Syringe	8.00	8.00
Red cell indices	20.00	20.00
DNA extraction	70.00	70.00
ARMS PCR	250.00	75.00
Electrophoresis	150.00	100.00
Photography	50.00	-
Sub total	548.00	273.00
Wastage @ 10%	55.00	27.00
Labour	200	200
Grand total	803.00 ($ 20)	500.00 ($ 12.5)

Policy-1:

Policy-1 is a situation in which all thalassaemics are provided adequate treatment facilities but no efforts are made for prevention. The cost of providing treatment to the estimated 5000 patients (N) born each year will be Rs. 800 million. The cost will increase at a rate equal to N x number of years from the start of policy. At ten years it will be Rs. 8.8 billion and at 20 years it will be Rs. 16.8 billion (Fig: 10.6).

Table: 8.8. Estimated cost of prenatal diagnosis for thalassaemia. The cost of termination is included at ¼ of the actual cost because only 1 in 4 would actually require termination.

Procedure:	No:	Rate (Rs.):		Cost (Rs.):	
		Standard PCR:	Multiplex PCR:	Standard PCR:	Multiplex PCR:
Mutation analysis	X 2	800	500	1600	1000
CVS:	X 1	-	-	200	200
Fetal testing	X 1	800	800#	800	800
Cost of termination:	X 1	-	-	1000	1000
Total	-	-	-	3600 ($ 90)	3000 ($ 75)

Fetal DNA is tested by the standard ARMS PCR.

Policy-2:

It is based on providing treatment facilities to the affected children as in Policy-1 but at the same time it also includes counselling of the affected couples. Alwan and Modell (1997) have suggested that when the final family size is large, if parents have no more pregnancies after the birth of one affected child, the birth rate of affected children, would fall by as much as 50%. This hypothesis, however, is subject to the availability as well as to the acceptance of family planning. A more realistic option would be to offer prenatal diagnosis to such couples.

If it is assumed that Policy-2, including retrospective counselling and the offer of prenatal diagnosis, would reduce the birth incidence of thalassaemia by about 50%, the number of children requiring treatment would also be reduced to half. The cost of providing prenatal diagnosis to 2N (N/2 X 4) number of couples would, however, be added to the net annual cost for the policy. The estimated total cost for the 1st year of the policy would be Rs. 830 million that includes Rs. 30 million for carrying out 10,000 prenatal diagnoses at the rate of Rs. 3000 per procedure. At 10th year of the policy the total annual expenditure would be Rs. 4.8 billion and at 20th year it would be Rs. 8.8 billion (Fig: 8.1).

Policy-3:

This includes all of the features of Policy-2 and in addition it also caters for prospective identification of couples at risk and the offer of prenatal diagnosis. Table 8.9 gives a

calculation of the number of individuals who may have to be screened if index thalassaemic families are targeted. The number of individuals requiring screening in the 0.225 million index families is estimated at 22 million (average 97 individuals per family). This would identify approximately 126,500 at risk couples and about 16% of this (20,000) would also require prenatal diagnosis. The total cost of screening 22 million people would be Rs. 1.7 billion (Table: 8.11).

The expected number of women who may require screening if approached through an antenatal clinic is 31 million (Table: 8.10). This would identify 5% (1.55 million) carrier women whose husbands would also require screening. The total number of individuals who may require screening would be approximately 32.5 million and the cost of screening would be Rs. 1.95 billion (Table: 8.11).

Table: 8.9. Calculation of the total number of couples and the number of at risk couples in a targeted screening approach.

• Population of Pakistan:	135 million
• Total number of carriers (at 5% carrier rate):	6.75 million
• Average number of individuals per family:	97 (Chapter: 5)
• Total number of families:	135 / 97 = 1.39 million
• Percent of carriers per family (Chapter: 5):	31%
• Number of carriers per family:	31/100 X 97 = 30
• Number of families at risk:	6.75 million / 30 = 0.225 million
• Average number of couples per family:	22.5
• Total number of couples in families at risk:	22.5 X 0.225 = 5.0625 million
• Proportion of consanguineous couples:	45% (2nd cousins or closer)
• Estimated proportion of couples at risk at: 45% consanguineous marriages (Chapter 5 & 9):	13 X 13 / 100 + 16 X 5 / 100 = 2.5%
• Total number of at risk couples:	2.5% of 5.0625 million = 126562

Table: 8.10. Calculation of the number of couples who may require antenatal screening.

• Proportion of females in active reproductive life:	46% (31 million)*
• Number of couples in active reproductive life: 31 million	
• Expected numbers where husband's screening may be required:	5% of 31 = 1.55 million
• Total numbers that may require screening:	31 + 1.55 = 32.55 million
• Total number of at risk couples identified:	5% of 1.55 = 77500

*Annual report of Health Services in Pakistan 1995-96.

Table: 8.11. The cost of family and antenatal screening for β-thalassaemia in Pakistan.

Approach:	No. of Individuals required to be screened: (millions)	No of at risk Couples identified:	Cost of screening for β-thalassaemia (Rs):	
			Per 1000:	Total:
Family:	21.8	126500	78,000	1.7 billion
Antenatal:	32.5	77,500*	60,000	1.95 billion

* The number of at risk couples identified by antenatal screening are less as compared to targeted approach because the later group also includes couples who are not in active reproductive life.

The cost of prospective identification of at risk couples, whether done by screening the index families or by antenatal screening, will be added to the net initial expenditure for the Policy-3. However, this can conveniently be spread over the first few years of the policy. The overall effect of Policy-3 is expected to reduce the number of births of affected children to approximately 90% of N (Alwan and Modell 1997). Consequently, the number of affected children requiring treatment would be reduced to 90%. But at the same time an additional cost of approximately 54 million for providing prenatal diagnosis (Rs. 3000 per diagnosis) to the at risk couples (90% of N x 4=18,000) would be added to the total cost for Policy-3. The initial cost of Policy-3 would be approximately Rs. 1.5 billion that will gradually increase over the next ten years to Rs. 2.88 billion and to Rs. 4.2 billion till the 20th year of the policy.

Comparison between the three policies:

There is a clear financial benefit in adopting Policy-2 and Policy-3 as compared to Policy-1. Policy-3 is obviously more cost effective than the Policy-2. The expenditure on Policy-3 would be higher during the initial few years that would be balanced by the huge amount of savings achieved in the years to follow. A basic assumption in the three policies discussed includes provision of adequate treatment facilities to all patients. An immediate benefit in adopting either Policy-2 or Policy-3 lies in the savings achieved on treatment of the affected children. Since the Government of Pakistan is not spending anything on treatment of thalassaemia, the immediate benefits of adopting a policy cannot be appreciated. However, it would certainly result in saving of the resources of non-governmental organizations.

9 Consanguineous marriage and recessive disorders

Introduction:

The term "consanguineous" literally means related by blood. A consanguineous marriage is defined as marriage between individuals who have at least one common ancestor usually not more than three generations back and the progeny of a consanguineous marriage is "inbred" (Bittles 1994). All human beings are relatives and our progenitors might even have been one single couple (Vogel and Motulsky 1986). There is some evidence to suggest that a relatively small founder population migrated out of Africa to give rise subsequently to all non African populations (Jones and Rouhani 1986). The long time period since divergence of human populations has helped several intervening mutations to cause genetic variability between different human beings. The relatedness of human beings assumes importance in the context of recessive mutations, as these are expressed only when inherited in a homozygous state. The chances of inheriting two identical genes, including neutral as well as pathological genes, at a particular locus are increased if the parents are close relatives (Bodmer and Cavalli-Sforza 1976).

Genetic effects of consanguineous marriage:

The main genetic consequence of consanguineous marriage is a reduction of genetic variation and an increase in the proportion of homozygotes. Recessive genes, that are unable to express in the heterozygous state, are thus brought to the fore (Bodmer and Cavalli-Sforza 1976). Increase in the proportion of homozygotes should uniformly affect the pathological, neutral as well as the beneficial traits. However, the effects of increased homozygosity for all genes are not distributed evenly about the population mean and a bias towards the pathological end of the spectrum may be observed (Modell and Kuliev 1992). There is no dearth of information on the association of consanguinity and increased incidence of genetic disorders (Neel and Schull 1962; Klingberg et al, 1971;

Basu 1975; Naderi 1979; Khlat et al, 1986; Bundey et al, 1991; Teebi 1994). A population inevitably carries a certain load of detrimental genes originating through mutations. Dominant genes are rapidly eliminated while recessive genes can be hidden and so can accumulate. Studies on the relationship between consanguinity and genetic disorders indicate that on an average an individual may be heterozygous for 1.4 lethal equivalents (Bittles and Neel 1994). The detrimental recessive genes may be unmasked by marriages between close relatives and this risk is directly proportional to the degree of relatedness between the mates (Bittles 1980).

The data on association of consanguineous marriages and harmful traits have almost completely overshadowed the beneficial traits, if any, that might be associated with consanguineous marriages. A prime example of the absence of inbreeding depression can be seen in the genealogies of the Egyptian pharaohs, in which brother-sister mating was practised for many generations without known or reported ill effects (Strickberger 1968). Some studies of intelligence suggest an association between consanguineous marriages and reduced cognitive performance (Slatis et al, 1961; Bashi 1977). Similarly, there is evidence to suggest higher gross fertility in a consanguineous mating (Bittles 1994).

Inbreeding estimates:

The genetic effects of consanguineous marriage and the resulting inbreeding are measured by a coefficient (F), first proposed by Sewall Wright (Strickberger 1968). The coefficient of inbreeding is the probability that an individual receives at a given locus two genes that are identical by descent (copies of a single gene carried by a common ancestor). A closely related coefficient of kinship (Φ) is the probability that a gene taken at random from an individual is identical by descent to a gene at the same locus taken at random from another individual (Vogel and Motulsky 1986). The difference between the two coefficients is that Φ applies to two individuals who have common ancestors and F applies to one individual and measures the degree of relationship between his/her parents.

The value of F for a first cousin marriage is 0.0625 i.e. 6.25% of the genes are identical by descent. In 1½ cousin and 2nd cousin marriage F is 0.0313 and 0.0156 respectively (Bodmer and Cavalli-Sforza 1976). In populations where consanguineous marriage is customary for several generations the estimates that do not take into account antecedent consanguineous marriages are expected to be less than the actual values (Bittles 1994). When extensive pedigree records are available and it is possible to go back for many more generations the value of F may be two or more times as large as obtained through the usual procedures (Bodmer and Cavalli-Sforza 1976).

Inbreeding estimates based on genetic polymorphism:

Genetic polymorphism may be defined as a trait encoded by a piece of DNA (a locus) with two or more alleles (sequence variants), of which at least two occur at a frequency of more than 1% in a given population (Vogel and Motulsky 1986). Blood groups and protein polymorphisms have been used in the past to estimate inbreeding in human populations (Workman and Niswander 1970). The estimates are based on the principle that in a population where mating is not random the heterozygotes are reduced in comparison to Hardy-Weinberg proportions while homozygotes are increased (Bodmer and Cavalli-Sforza 1976). The extent of inbreeding in such a population can be measured by quantifying the departure from Hardy Weinberg proportions of the frequencies of polymorphic genes.

Molecular genetic analysis has revealed that about 2% of the total human genome encodes proteins and the rest does not appear to have any sequence dependent function. A bulk of the intergenic DNA is unique because it consists of tandemly repeating sequences whose length may vary between different individuals in the same population. The polymorphisms created by such elements are termed variable number of tandem repeats (VNTR) for the larger repeats and short tandem repeats (STR) for 1-6 base pair repeats (Krawczak and Schmidtke 1994). VNTRs and STRs follow the same rules of inheritance as the remainder of DNA and therefore are ideal sites as polymorphic genetic markers (Housman 1995).

VNTRs and STRs can be analyzed by the Polymerase Chain Reaction (Horn et al, 1989; Urquhart et al, 1994). The STRs offer a better choice as compared to the VNTR loci because the larger allele of a VNTR system may be missed in PCR based amplification (Newton and Graham 1994). The STRs have an added advantage of accurate sizing of the alleles on automated equipment (Smith 1995). The STR allele frequencies have a potential for use in estimating the extent of inbreeding in a population. However, there are no published data on the use of STR allele frequencies for inbreeding estimates.

Objectives of the study:

This chapter describes the pattern and frequency of consanguineous marriages and their relationship to autosomal recessive disorders in Pakistani families. The effect that an antecedent consanguineous marriage might have on the inbreeding estimates is also studied. In addition, an attempt is made to calculate the F values in a population by using polymorphic STR allele frequencies. The same STR was also studied in the successive generations of two large families to highlight the genetic effects of consanguineous marriage.

Material and methods:

The study families:

A total of 14 large families were studied including nine with a known history of haemoglobin disorder (subjects) and five without such history (controls). The characteristics of the families studied are described in Chapter 5 (Tables: 5.2 and 5.3).

Drawing pedigrees:

Pedigree information on at least the last three generations in each family was collected. Information on relationships of the more remote ancestors was also included where available. The information was obtained from a well informed person in the family. Information about the 2nd and 3rd generations was readily available. However, it was more difficult to get information about the 1st generation or earlier. In the urban families pedigree information had to be collected from multiple sources because the members had

infrequent contact with each other. Best efforts were made to ensure the reliability of information.

The pedigree was initially drawn as a sketch on paper. Later, this information was transferred to a computer by using the software package "Cyrillic Version 2.00" (The Magdalen Centre, Oxford).

Morbidity and mortality in the family:

Information about the births of children with major congenital malformations or symptoms suggestive of a genetic disorder was collected from the person who provided the pedigree information. The result of screening for β-thalassaemia, where carried out, was also included in the pedigree.

Consanguineous marriages:

The couples were classified as double first cousins (F=0.1250), first cousins (F=0.0625), first cousins once removed (1½ cousins) (F=0.0313), and second cousins (F=0.0156).

Coefficient of inbreeding (F) and coefficient of kinship (Φ):

The coefficient of inbreeding of each individual and coefficient of kinship for each couple in a family was calculated using Cyrillic version 2.00. The value of F and Φ for individuals in one of the families was also calculated by the manual method of path coefficient (Vogel and Motulsky 1986). All common ancestors were marked and the mates were connected by all possible pathways leading to the common ancestors. The number of steps in each path was counted. The F values were calculated by the following formula:

$$F = \tfrac{1}{2}\left(2^{-m_1} + 2^{-m_2} \ldots + 2^{-m_r}\right) = \tfrac{1}{2} \sum_{i=1}^{r} 2^{-m_i}$$

where m denotes the number of steps connecting an individual with the respective common ancestor. The average values of F and Φ in a generation of a family were calculated by the following formulae:

$$F=\sum p_i F_i$$

$$\Phi=\sum p_i \Phi_i$$

The summations go over various types of consanguineous marriages, with p_i , F_i and Φ_i being the relative frequency, coefficient of inbreeding and coefficient of kinship of the i-th type of consanguineous marriage (Vogel and Motulsky 1986).

Short tandem repeat (STR) analysis:

Allele frequencies for a polymorphic DNA marker, D21S11 (Sharma and Litt 1992), were used to calculate the coefficient of inbreeding in a randomly selected population sample of 132 unrelated individuals with β-thalassaemia trait. They presented at the Department of Haematology, Armed Forces Institute of Pathology, Rawalpindi, for screening of thalassaemia. The parental consanguinity of the subjects was also noted. A comparison between the F obtained by STR allele frequencies and the conventional method was made.

D21S11 genotypes were determined for the available members of two subject families. A total of 140 individuals from Family No: 1 and 85 individuals from Family No: 2 were studied. The genotypes of 5 dead members in the two families were inferred from their children. The data on genotypes in various generations were used to investigate the effect of genetic drift in the successive generations. The allele frequencies in each generation were calculated by pooling together all individuals in that particular generation because they were the people who would be passing on their alleles to the next generation.

Theoretical considerations:

Gene frequency can be determined by simply counting the alleles and by remembering that every individual has two copies of each gene (Bodmer and Cavalli-Sforza 1976). At a di-allelic locus with 5% carrier rate of β-thalassaemia, for example, the thalassaemia gene frequency will be 0.025 (5 β-thalassaemia genes out of a total of 200 genes at that locus). A multi-allelic system like STRs gives rise to many genotypes. The frequency of each

STR allele can also be determined by the method of counting alleles. Each heterozygote represents one gene and the homozygote represents two genes.

In a randomly mating population, the relation between allele and genotype frequencies is simple. If the gene frequencies are known the Hardy-Weinberg law can predict the corresponding genotype frequencies (Vogel and Motulsky 1986). At a di-allelic locus, for example, if the frequency of allele *A* is *p* and that of *B* is *q* then the genotype *AA*, *AB*, and *BB* will have frequencies of p^2, *2pq*, and q^2 respectively. The same rule can be extended to predict the genotype frequencies at a multi-allelic locus (Committee on DNA technology in forensic science 1992). In a five allele system, for example, if the alleles *A*, *B*, *C*, *D*, and *E* have frequencies *p*, *q*, *r*, *s*, and *t* respectively then the 15 possible genotype frequencies (predicted by the formula [n(n+1)/2] where n is the number of alleles), would be p^2, q^2, r^2, s^2, and t^2 for the homozygotes, and *2pq*, *2pr*, *2ps*, *2pt*, *2qr*, *2qs*, *2qt*, *2rs*, *2rt*, *2st* for the heterozygotes.

In a population where mating is not random, the proportion of heterozygotes is reduced in comparison to Hardy-Weinberg proportions by an amount *2Fpq*, while that of each homozygote is increased by *Fpq*. Where *F* is the inbreeding coefficient, and *p* and *q* are the frequencies of the alleles under consideration (Bodmer and Cavalli-Sforza 1976). The genotypic proportions at a single di-allelic locus, say (*AA*, *AB*, *BB*) = (*P*, *H*, *Q*), where $P+H+Q=1$, can be represented in terms of the gene frequencies of *A* and *B*, denoted by *p* and *q*, by the formulas:

$$\underset{P = p^2 + pqF;}{AA} \qquad \underset{H = 2pq\,(1 - F);}{AB} \qquad \underset{Q - q^2 + pqF}{BB}$$

where $p = P + \frac{1}{2}H$, $q = 1 - p = Q + \frac{1}{2}H$, and $F = 1 - H / 2pq$. Here *F* denotes the deviation from Hardy-Weinberg proportions due to the joint effect of all forces acting on the pattern of genetic variation, including non-random mating, selection, and mutation. When inbreeding is the only factor, *F* is identical with the classical inbreeding coefficient (Workman and Niswander 1970).

Calculation of gene frequencies and coefficient of inbreeding:

The gene frequencies for the D21S11 STR alleles were calculated by the simple method of counting. The F value in the subject population was estimated by the following formula:

$$F = 1 - H / 2pq$$

where H is the observed proportion of heterozygotes for the allele whose gene frequency is represented by p, and q=1-p.

Results:

Characteristics of the families:

Characteristics of the 14 families studied were described in Chapter 5 (Table: 5.2 and 5.3). There were 9 Punjabi and 5 Pathan families. Family Nos: 1 and 2 were from the urban areas whereas all others were from the rural areas.

Drawing pedigrees:

The individuals from the families were divided into generations and only the last three generations were taken into consideration. The third last generation in a family was called 1st generation and the subsequent generations were labelled 2nd and 3rd. Most pedigrees had complex consanguineous relationships, therefore drawing a pedigree was also complex. The commonest problem was to keep all individuals in a particular generation at the same level. In most situations it was not possible without keeping the individuals in the same generation at split-levels. Another problem was in assigning a generation to the couple and the children of 1½ cousins. The couple were arbitrarily placed (counted) in the generation of the parent from the senior generation. Their children were placed (counted) in the generation of the parent from the younger generation. However, while counting the number of individuals in a generation, each individual was counted in the generation to which it actually belonged. Pedigrees of the 9 subject and 5 control families are presented in Figures 9.4-9.17.

Pattern of marriages:

There were 319 couples in the three generations of 14 families (Table: 9.1). This included 195 couples in the nine subject families and 124 in the five control families. There were 1.3% double first cousins, 33.2% 1st cousins, 4.1% 1½ cousins, and 3.8% 2nd cousins. There were no statistically significant differences between the pattern of marriages in the subject and the control families. In 319 couples 165 (51.7%) were unrelated but from the same Biradri/Tribe and only 19/319 (6.0%) couples were completely unrelated.

It was noted that consanguineous marriages were more frequent in the 2nd generation than the 1st (Table: 9.2). The frequency of 1st cousin marriages had increased from 12% in the 1st generation to 45% in the 2nd generation (p=<0.0001). There were 12 married couples in the 3rd generation and 7 of them were consanguineous. Although its difference from the previous generation is statistically insignificant due to small numbers but the trend is obvious. Consanguineous marriages have reduced Biradri marriages from 71% in the 1st generation to 40% in the 2nd generation (p=0.003). The frequency of consanguineous and Biradri marriages was similar in the subject as well as the control families. However, in the 2nd generation there were 51% 1st cousin couples in the subject families as compared to 37.5% in the controls, but the difference was statistically insignificant (p=0.25).

Calculation of F and Φ:

The procedure of calculating F and Φ by manual method was very complicated and time consuming. On the other hand the use of Cyrillic 2.00 was very efficient in calculating F and Φ in the complex pedigrees. A complete correlation between the manual and the computerized method was found.

Coefficient of inbreeding (F):

F for the three generations of the subject families is presented in Table: 9.3. A progressive increase for the 1st (0.0046), 2nd (0.0128) and 3rd (0.0352) generation was observed. There were no significant differences between F in the three generations of the subject and the control families. There was a wide variation of F between the individual families. In the 3rd generation, for example, it varied from 0.000 to 0.0782.

Coefficient of kinship (Φ):

Observed Φ of consanguineous couples in the 2nd generation showed a value of 0.1346 for a double first cousin marriage, 0.0718 for a 1st cousin marriage, 0.0330 for a 1½ cousin marriage and 0.0242 for a 2nd cousin marriage (Table: 9.4). It was noted that the differences between the observed and the expected value of F for the close and the distant consanguineous relationships was similar.

Genetic/congenital disorders in the study families:

Haemoglobin disorders:

There were 170/571 (30%) carriers in the subject families (Table: 9.5). The number of carriers in each generation varied from 30-34%. In the 2nd generations there were 83/251 (33%) carriers and 30 were unmarried (30% of 101 unmarried individuals). In 150 married individuals in 2nd generation 53 (35.3%) were carriers. In the 2nd generation 32/150 (21%) carriers were married to a 1st cousin, 3/150 (2%) to a 1½ cousin, 2/150 (1.3%) to a 2nd cousin and the remaining 16/150 (10.7%) to an unrelated Biradri member. In 8/37 (21.6%) carriers married to a cousin the spouse was also a carrier. However, the spouses of 4/16 (25%) carriers married to an unrelated Biradri/Tribe member were also carriers. No carrier was married to a carrier from a different Biradri/Tribe.

Correlation between the number of carriers, coefficient of kinship and at risk couples:

Table 9.6 summarizes the number of carriers, mean coefficient of kinship, and the number of at risk couples in the 1st and 2nd generations of the subject (index) families. The 3rd generation did not have enough couples so it was not included in the comparison. The proportion of carriers in the 1st generation of most families was lower than in the 2nd generation. Similarly, coefficient of kinship was also lower in the 1st generation. This correlated with the absence of at risk couples in this generation. Family No: 10, however, had 45% carriers and in spite of a low Φ two at risk couples were seen. Both of the couples although unrelated were from the same Tribe. In the 2nd generation the number of at risk couples generally correlated with the number of carriers and the mean Φ for the generation. In most families the carrier rate was above 30% and the kinship coefficient was also high. In Family No: 1 four at risk couples were seen in spite of a relatively low Φ (0.0303). However, the carrier rate in the generation was fairly high (33%) and three non-

consanguineous at risk couples were from the same Biradri all of whom also had the same β-thalassaemia mutation (IVSI-1). In Family No: 8, no at risk couple was seen. Although the carrier rate in the individuals tested was fairly high (29%) but the mean kinship coefficient was low (0.0208). In Family No: 9 in spite of a low carrier rate and low Φ one at risk couple was present. The spouses were unrelated but were from the same Tribe. In Family No: 10 carrier rate (22%) as well as Φ (0.0313) were low and there was no at risk couple in this generation.

Couples at risk of genetic disorders:

In the nine subject families 12/195 (6.2%) couples had affected children and 2/195 (1%) were at risk but did not have an affected child (Table: 9.7). Out of the 79 consanguineous couples 11 (14%) had an affected child as compared to 3/116 (2.5%) amongst the non-consanguineous couples (p=0.005). In Family No: 1, four couples were found to have thalassaemic children. However, the spouse of only one was a first cousin (Φ=0.0869) whereas in the other three spouses were unrelated Biradri members. Mutation analysis revealed that all four couples had the same β-thalassaemia mutation (IVSI-1 (G-T). In Family No: 2, in addition to the index couple (Φ=0.0469), screening for thalassaemia resulted in detection of one more at risk couple (Φ=0.1210). The couple did not have an affected child. In the same family two other consanguineous couples (Φ=0.0820 and 0.0781) had children with congenital deafness. In Family Nos: 5 and 10, one at risk couple each (Φ=0.0781 and 0.000), in addition to the index couples, were identified as a result of screening for a haemoglobin disorder. Both of the couples did not have an affected child.

In the five control families 4/124 (3.2%) couples had children possibly affected by a congenital or genetic disorder (Table: 9.7). All 4 couples were consanguineous and they formed 7% of the 56 consanguineous couples. In contrast, none of the 68 non-consanguineous couples had children possibly affected by a genetic or congenital disorder (p=0.03). In Family No: 11 one consanguineous couple (Φ=0.0781) had three children that died during the first month of life due to unknown causes. In Family No: 13 two consanguineous couples (Φ=0.0625) had several children that died immediately after birth

due to unknown causes. In Family No: 14 one couple (Φ=0.1328) was found to have deaths of two children due to multiple congenital abnormalities. In Family No: 15 five consanguineous couples had children who died between 1-5 years of age. The cause of death in all such children was not similar. There was also no visible evidence to suggest that they had any congenital malformation. The possibility of a genetic disorder could not be ruled out.

Table: 9.1. Pattern of marriages in the three generations of the 14 families studied.

Family:	DIC	1st cousins	1½ cousins	2nd cousins	Biradri	Non-Biradri	Total
Subject Families:							
No: 1	-	15	2	3	30	2	52
No: 2	-	13	1	1	4	2	21
No: 3	-	7	-	1	5	1	14
No: 4	-	6	-	-	4	2	12
No: 5	-	8	-	-	9	2	19
No: 6	-	5	-	-	9	-	14
No: 8	-	6	2	-	15	5	28
No: 9		5	-	1	14	-	20
No: 10	-	3	-	-	12	-	15
Sub total	-	68 (34.9%)	5 (2.6%)	6 (3.1%)	102 (52.3%)	14 (7.2%)	195 (100%)
Control families:							
No: 11	-	10	2	1	9	-	22
No: 12	-	-	-	-	11	-	11
No: 13	-	6	-	-	16	1	23
No: 14	3	10	3	3	13	-	32
No: 15	1	12	3	2	14	4	36
Sub total	4 (3.2%)	38 (30.6%)	8 (6.6%)	6 (4.8%)	63 (50.8%)	5 (4.0%)	124 (100%)
Grand total	4 (1.3%)	106 (33.2%)	13 (4.1%)	12 (3.8%)	165 (51.7%)	19 (6.0%)	319 (100%)

DIC: double first cousins.

Table: 9.2. Pattern of marriages in the successive generations of the nine subject and five control families.

Family:	Number of couples																			
	1st generation							2nd generation							3rd generation						
	Consanguineous				Unrelated		All	Consanguineous				Unrelated		All	Consanguineous				Unrelated		All
	DIC	1st	1½	2nd	Bir	NB		DIC	1st	1½	2nd	Bir	NB		DIC	1st	1½	2nd	Bir	NB	
Subject Families:																					
No: 1	-	1	-	2	11	2	16	-	13	2	1	17	-	33	-	1	-	-	2	-	3
No: 2	-	5	1	-	4	2	12	-	8	-	1	-	-	9	-	-	-	-	-	-	-
No: 3	-	1	-	-	3	1	5	-	6	-	1	2	-	9	-	-	-	-	-	-	-
No: 4	-	-	-	-	4	2	6	-	6	-	-	-	-	6	-	-	-	-	-	-	-
No: 5	-	2	-	-	4	1	7	-	6	-	-	5	1	12	-	-	-	-	-	-	-
No: 6	-	1	-	-	7	-	8	-	4	-	-	2	-	6	-	-	-	-	-	-	-
No: 8	-	-	-	-	5	3	8	-	4	2	-	7	2	15	-	2	-	-	3	-	5
No: 9	-	-	-	-	10	-	10	-	4	-	2	4	-	10	-	-	-	-	-	-	-
No: 10	-		-	-	9	-	9	-	3	-	-	3	-	6	-	-	-	-	-	-	-
Sub total	-	10	1	2	57	11	81	-	54	4	5	40	3	106	-	3	-	-	5	-	8
%		12.3	1.3	2.5	70.3	13.5	100		50.9	3.8	4.7	37.7	2.8	100							
Control Families:																					
No: 11	-	1	-	-	4	-	5	-	9	2	1	5	-	17	-	-	-	-	-	-	-
No: 12	-	-	-	-	5	-	5	-	-	-	-	6	-	6	-	-	-	-	-	-	-
No: 13	-	-	-	-	6	1	7	-	6	-	-	10	-	16	-	-	-	-	-	-	-
No: 14	-	1	-	2	4	-	7	3	5	3	1	9	-	21	-	4	-	-	-	-	4
No: 15	-	2	2	-	10	2	16	1	10	1	2	4	2	20	-	-	-	-	-	-	-
Sub total	-	4	2	2	29	3	40	4	30	6	4	34	2	80	-	4	-	-	-	-	4
%		10.0	5.0	5.0	72.5	7.5	100	5.0	37.5	7.5	5.0	42.5	2.5	100							
Grand total	-	14	3	4	86	14	121	4	84	10	9	74	5	186	-	7	-	-	5	-	12
%		11.6	2.5	3.3	71.1	11.6	100	2.1	45.2	5.4	4.8	39.8	2.7	100		58.3			41.7		100

DIC: double first cousins; Bir: Biradri member; NB: Non-Biradri member.

Table: 9.3. Coefficient of inbreeding (F) in the three generations of the nine subject and five control families.

Family:	Coefficient of Inbreeding (F):								
	1st generation:			2nd generation:			3rd generation:		
	Number:	Average:	Range:	Number:	Average:	Range:	Number:	Average:	Range:
Subject Families:									
No: 1	32	0.0122	0.000-0.0625	75	0.0153	0.000-0.0664	102	0.0246	0.000-0.0869
No: 2	20	0.0013	0.000-0.0625	48	0.0360	0.000-0.0820	23	0.0782	0.0469-0.1210
No: 3	11	0.0071	0.000-0.0625	28	0.0211	0.000-0.0625	24	0.0533	0.0000-0.0781
No: 4	12	0.000	0.000-0.000	19	0.000	0.000-0.000	20	0.0625	0.0625
No: 5	14	0.0067	0.000-0.0625	31	0.0297	0.000-0.0625	28	0.0441	0.000-0.1094
No: 6	16	0.000	0.000-0000	27	0.0093	0.000-0.0625	20	0.0500	0.000-0.0625
No: 8	13	0.0072	0.000-0.0625	33	0.0009	0.000-0.0625	77*	0.0231	0.000-0.0625
No: 9	21	0.000	0.000-0.000	28	0.000	0.000-0.000	42	0.0245	0.000-0.0625
No: 10	17	0.000	0.000-0.000	42	0.000	0.000-0.000	18	0.0139	0.000-0.0625
Sub total	156	0.0058	0.000-0.0625	331	0.0141	0.000-0.0820	354	0.0343	0.000-1210
Control Families:									
No: 11	12	0.0065	0.000-0.0625	34	0.0165	0.000-0.0625	84	0.0415	0.000-0.0781
No: 12	10	0.000	0.000-0.000	25	0.000	0.000-0.000	34	0.000	0.000-0.000
No: 13	14	0.0011	0.000-0.0156	37	0.0017	0.000-0.0625	74	0.0203	0.000-0.0625
No: 14	14	0.000	0.000-0.000	42	0.0123	0.000-0.0625	100*	0.0237	0.000-0.1328
No: 15	33	0.0019	0.000-0.0313	65	0.0149	0.000-0.0625	74	0.0576	0.000-0.1406
Sub total:	83	0.0019	0.000-0.0625	203	0.0105	0.000-0.0625	366	0.0360	0.000-0.1406
Grand total:	239	0.0046	0.000-0.0625	534	0.0128	0.000-0.0820	720	0.0352	0.000-0.1406

* combined total of the 3rd and 4th generations

Table: 9.4. Coefficient of kinship (Φ) for consanguineous couples in the 2nd generation of the study families.

Family:	Coefficient of kinship (Φ):											
	Double 1st cousins (0.1250):			1st cousins (0.0625):			1½ cousins (0.0313):			2nd cousins (0.0156):		
	n:	Average:	Range:	n:	Average:	Range:	n:	Average:	Range:	n:	Average:	Range:
No: 1	-	-	-	13	0.0713	0.0625-0.0869	2	0.0332	0.0322-0.0341	1	0.0176	0.0176
No: 2	-	-	-	8	0.0995	0.0781-0.1210	-	-	-	1	0.0469	0.0469
No: 3	-	-	-	6	0.0729	0.0625-0.0781	-	-	-	1	0.0156	0.0156
No: 4	-	-	-	6	0.0625	0.0625	-	-	-	-	-	-
No: 5	-	-	-	6	0.0833	0.0781-0.1094	-	-	-	-	-	-
No: 6	-	-	-	4	0.0625	0.0625	-	-	-	-	-	-
No: 8	-	-	-	4	0.0625	0.0625	2	0.0313	0.0313	-	-	-
No: 9	-	-	-	4	0.0625	0.0625	-	-	-	1	0.0156	0.0156
No: 10	-	-	-	3	0.0625	0.0625	-	-	-	-	-	-
No: 11	-	-	-	9	0.0729	0.0625-0.0781	2	0.0313	0.0313	1	0.0313	0.0313
No: 12	-	-	-	-	-	-	-	-	-	-	-	-
No: 13	-	-	-	6	0.0625	0.0625	-	-	-	-	-	-
No: 14	3	0.1328	0.1328	5	0.0680	0.0625-0.0781	3	0.0332	0.0332	1	0.0195	0.0195
No: 15	1	0.1406	0.1406	10	0.0758	0.0625-0.0937	1	0.0391	0.0391	2	0.0234	0.0234
Observed Φ	4	0.1348	0.1328-0.1406	84	0.0718	0.0625-0.1210	10	0.0330	0.0313-0.0391	8	0.0242	0.0156-0.0469
Expected Φ		0.1250			0.0625			0.0313			0.0156	
Obs/Exp Φ		1.078			1.149			1.054			1.551	
Obs-Exp Φ		0.0098			0.0093			0.0017			0.0086	

n: number of couples

Table: 9.5. Summary of the results of screening for haemoglobin disorders in the available members of the nine subject families with a history of haemoglobin disorder.

Family:	Members:		Carriers:								
	Alive:	Tested:	1st Generation:	2nd Generation:						3rd Generation#:	Total:
				Married to:				Unmarried:	Total:		
				1st C:	1½ C:	2nd C:	Biradri:				
No: 1	199	138 (69%)	2/9	7/47	1/47	-	8/47	2/7	18/54 (33%)	16/75 (21%)	36/138 (26%)
No: 2	85	85 (100%)	4/17	6/19	1/19	1/19	1/19	7/28	16/47 (34%)	6/21 (27%)	26/85 (31%)
No: 3	55	51 (93%)	1/6	3/15	0/15	0/15	0/15	4/9	7/24 (29%)	6/21 (29%)	14/51 (27%)
No: 4	48	41 (85%)	3/6	6/11	0/11	0/11	0/11	2/7	8/18 (44%)	7/17 (41%)	18/41 (44%)
No: 5	69	45 (65%	2/5	5/13	0/13	0/13	1/13	2/6	8/19 (42%)	7/21 (33%)	17/45 (38%)
No: 6	60	42 (70%)	4/9	4/8	0/8	0/8	1/8	5/14	10/22 (46%)	5/11 (46%)	19/42 (45%)
No: 8	98	58 (59%)	1/3	0/14	1/14	0/14	2/14	2/4	5/18 (28%)	6/37 (16%)	12/58 (21%)
No: 9	79	48 (61%)	3/7	0/12	0/12	1/12	2/12	1/2	4/14 (29%)	5/27 (19%)	11/48 (23%)
No: 10	73	63 (86%)	5/11	1/11	0/11	0/11	1/11	5/24	7/35 (21%)	5/17 (29%)	17/63 (27%)
Total:	766	571 (75%)	25/73 (34%)	32/150 (21.3%)	3/150 (2.0%)	2/150 (1.3%)	16/150 (10.7%)	30/101 (29.7%)	83/251 (33%)	63/247 (26%)	170/571 (30%)

almost all of the carriers in the 3rd generation were unmarried

Table: 9.6. Correlation between the number of carriers, coefficient of kinship (Φ) and the number of at risk couples in individuals tested for a haemoglobin disorder in two generations of the families with history of haemoglobin disorder.

Family:	1st Generation (individuals tested):			2nd generation (individuals tested):		
	Carriers:	Φ:	At risk couples:	Carriers:	Φ:	At risk couples:
No:1	2/9	0.0059	None	18/54 (33%)	0.0303	4
No: 2	4/17	0.0300	None	16/47 (34%)	0.0937	2
No: 3	1/6	0.0125	None	7/24 (29%)	0.0503	1
No: 4	3/6	0.000	None	8/18 (44%)	0.0625	1
No: 5	2/5	0.0179	None	8/19 (42%)	0.0416	2
No: 6	4/9	0.0078	None	10/22 (45%)	0.0416	1
No: 8	1/3	0.000	None	5/18 (28%)	0.0208	None
No: 9	3/7	0.000	None	4/14 (29%)	0.0281	1
No: 10	5/11	0.000	2	7/35 (20%)	0.0313	None
Total:	25/73 (34%)	0.0082	2	83/251 (33%)	0.0445	12

Table: 9.7. Genetic/congenital disorders in the 14 study families.

Family:	Couples:										
	All:			With a genetic/congenital disorder:				Prospectively found at risk:			
	Con:	NC:	Total:	Con:	NC:	Total:	Condition:	Con:	NC:	Total:	Condition:
Subject (index) families:											
No: 1	20	32	52	1	3	4	Thalassaemia	-	-		-
No: 2	15	6	21	1 2	- -	1 2	Thalassaemia Congenital deafness	1	-	1	Thalassaemia
No: 3	8	6	14	1	-	1	Thalassaemia	-	-	-	-
No: 4	6	6	12	1	-	1	Thalassaemia	-	-	-	-
N0: 5	8	11	19	2	-	2	Thalassaemia	-	-	-	-
No: 6	5	9	14	1	-	1	Thalassaemia	-	-	-	-
No: 8	8	20	28	-	-	-	-	-	-	-	-
No: 9	6	14	20	1	-	1	Sickle	-	-	-	-
No: 10	3	12	15	1	-	1	Sickle	-	1	1	Sickle
Subtotal:	79	116	195	11/79 13.9%	3/116 2.5%	14/195 7.2%	-	1	1	2	-
Control families:											
No: 11	13	9	22	1	-	1	Unknown causes of deaths during the first month of life.	-	-	-	
No: 12	-	11	11	-	-	-		-	-	-	-
No: 13	6	17	23	2	-	2	Unknown causes of deaths at the time of birth.	-	-		
No: 14	19	13	32	1	-	1	Multiple congenital malformations.	-	-		
No: 15	18	18	36	??	-	??	??	-	-		-
Subtotal:	56	68	124	4/56 7.1%	0/68 0%	4/124 3.2%	-	-	-		
Grand total:	135	184	319	15/135 11.1%	3/184 1.6%	18/319 5.6%	Various	2	-		-

Con: Consanguineous; NC: Non-consanguineous

Short tandem repeat (STR) analysis:

D21S11 allele frequencies:

Ten alleles were seen at the D21S11 locus (Fig 9.1a). The alleles were named according to the size in base pairs as determined by an automated fragment size analyzer. Sharma and Litt (1992) showed that the alleles at D21S11 locus are four base pair repeats. It was observed that, in addition to the four base pair repeats, alleles varying by two base pairs were also present. Similar observations have also been made by other workers (Kimpton et al, 1993; Urquhart et al, 1994).

Table: 9.8 shows the overall genotype frequencies in 132 unrelated individuals. There were 36 different genotypes as compared to the theoretically predicted 55. The observed number of homozygotes for all alleles was 26. The observed frequencies for the common alleles (Table: 9.8) in the descending order were 234 (0.193), 220 (0.174), 224 (0.159), 216 (0.152), and 230 (0.144).

Calculation of F by the STR allele frequencies:

The F value, calculated by parental consanguinity of the 132 subjects, was found to be 0.0257 (Table: 9.9). The values of F as calculated by the frequency of the D21S11 alleles, for which a homozygote was also observed, are presented in Table: 9.10. The values ranged from 0.0231 with the commonest allele (234) to 0.0313 for the less common allele (230). The value of F calculated from the uncommon allele frequencies were widely outside the acceptable range (0.0567 and 0.1285 for the allele 238 and 228 respectively). The average F calculated from the five common alleles was 0.0272.

D21S11 analysis in the two subject families:

The results of D21S11 allele frequencies in the last three generations of Families No: 1 and 2 are shown in Table: 9.11 and 9.12 respectively. A considerable fluctuation in the frequency of different alleles in various generations was observed. The frequency of allele 220 in Family No: 1, for example, had progressively gone down from 0.20 to 0.120 to 0.052 in the last generation. The allele 224 in the same family, on the other hand, had increased in frequency from 0.05 to 0.102 to 0.132 in the last generation. Similar

fluctuations of the allele frequencies in various generations of Family No: 2 were also observed. The allele 220 had increased from 0.125 to 0.149 to 0.214 in the three successive generations. The allele 224 showed a progressive decline in its frequency from 0.20 to 0.149 to 0.095. Similarly the allele 230 was reduced from 0.10 to a complete extinction in the last generation.

Table: 9.8. D21S11 observed genotype frequencies in 132 unrelated individuals.

212	-									
216	-	3 (0.0227)								
220	-	8 (0.0606)	5 (0.0378)							
224	2 (0.0151)	10 (0.0757)	6 (0.0454)	4 (0.0303)						
226	-	3 (0.0227)	-	1 (0.0075)	-					
228	-	1 (0.0075)	2 (0.0151)	2 (0.0151)	-	1 (0.0075)				
230	-	5 (0.0378)	8 (0.0606)	4 (0.0303)	1 (0.0075)	-	4 (0.0303)			
234	-	4 (0.0303)	7 (0.0530)	6 (0.0454)	1 (0.0075)	2 (0.0151)	10 (0.0757)	7 (0.0530)		
238	1 (0.0075)	3 (0.0227)	5 (0.0378)	2 (0.0151)	1 (0.0075)	1 (0.0075)	-	5 (0.0378)	2 (0.0151)	
242	-	-	-	1 (0.0075)	-	-	2 (0.0151)	2 (0.0151)	-	-
	212	**216**	**220**	**224**	**226**	**228**	**230**	**234**	**238**	**242**

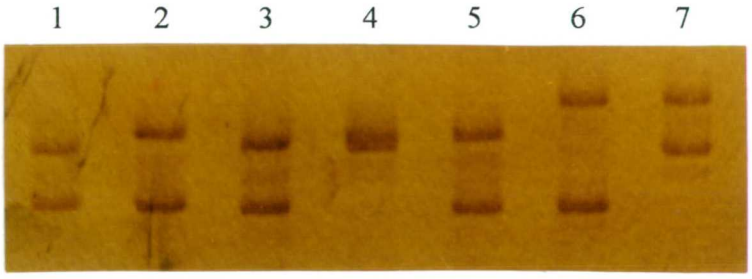


Fig: 9.1a . Results of the PCR amplification of the STR at D21S11 locus after electrophoresis on 6% denaturing polyacrylamide gel and silver staining. Lane 1 shows allele 216/226, lane 2: 216/228, lane 3: 216/226, lane 4: 226/226, lane 5: 216/228, lane 6: 216/234, and lane 7: 226/234. (Note: The same samples were initially run on ALF for allele sizing).

Table: 9.9. Coefficient of inbreeding of the 132 subjects calculated by the standard methods.

Consanguinity:	Number:	Percent:	Coefficient of Inbreeding:
1st Cousins:	45	34.1%	0.0212
1½ Cousins:	15	11.4%	0.0035
2nd Cousins:	9	6.8%	0.0010
Unrelated:	63	47.7%	0.0000
Total:	132	100%	0.0257

Table: 9.10. Calculation of *F* values from the D21S11 allele frequencies in a sample of 132 unrelated individuals.

Allele (n):	*p*	*q*=1-*p*	*H:*	1-*H:*	*2pq:*	*F*=1-*H* /*2pq*
212 (3)	0.011	-	-	-	-	-
216 (40)	0.152	0.848	34 (0.2575)	0.7425	0.2578	0.0288
220 (46)	0.174	0.826	36 (0.2727)	0.7273	0.2874	0.0253
224 (42)	0.159	0.841	34 (0.2575)	0.7425	0.2674	0.0277
226 (7)	0.027	-	-	-	-	-
228 (10)	0.038	0.962	8 (0.0606)	0.9394	0.0731	0.1285
230 (38)	0.144	0.856	30 (0.2272)	0.7728	0.2465	0.0313
234 (51)	0.193	0.807	37 (0.2803)	0.7197	0.3115	0.0231
238 (22)	0.083	0.917	18 (0.1363)	0.8637	0.1522	0.0567
242 (5)	0.019	-	-	-	-	-
All (264)	1.00	-	-	-	-	-

p= allele frequency; *H*= observed number of heterozygotes

Table: 9.11. D21S11 allele frequencies in the successive generations of Family No: 1.

Alleles:	1st generation: (*F*=0.0122)	2nd generation: (*F*=0.0153)	3rd generation: (*F*=0.0246)
212	1 (0.05)	1 (0.009)	-
216	4 (0.20)	30 (0.278)	39 (0.257)
220	4 (0.20)	13 (0.120)	8 (0.052)
224	1 (0.05)	11 (0.102)	20 (0.132)
228	1 (0.05)	6 (0.056)	11 (0.072)
230	5 (0.25)	30 (0.278)	45 (0.296)
234	4 (0.20)	16 (0.148)	28 (0.184)
238	-	1 (0.009)	1 (0.007)
Total:	20 (1.00)	108 (1.00)	152 (1.00)

Table: 9.12. D21S11 allele frequencies in the successive generations of Family No: 2.

Alleles:	**1st generation: (*F*=0.0125)**	**2nd generation: (*F*=0.0360)**	**3rd generation: (*F*=0.0782)**
216	8 (0.20)	23 (0.245)	10 (0.238)
220	5 (0.125)	14 (0.149)	9 (0.214)
224	8 (0.20)	14 (0.149)	4 (0.095)
226	5 (0.125)	7 (0.075)	8 (0.190)
230	4 (0.10)	10 (0.106)	-
234	9 (0.225)	22 (0.255)	11 (0.117)
238	1 (0.025)	2 (0.021)	-
Total:	40 (1.00)	94 (1.00)	42 (1.00)

Discussion:

The origin of consanguineous marriage on the Indian Subcontinent is not clear. No one knows what the conventions of the original population of India were, but the lower castes and tribal populations may well have favoured consanguineous marriage (Reddy 1994). The population of North India, however, was originally exogamous as much of North India is today (Sinclair 1972). Islam came to the Subcontinent with the arrival of Arabs and Turks in the 7th and 8th century A.D (Wolpert 1977). The practice of consanguineous marriages amongst the Muslims might be related to adoption of Islam. As it was predominantly the lower castes who were attracted by the Islamic concept of human equality, some converts might already have favoured consanguineous marriage (Modell and Kuliev 1992).

Frequency and trends of consanguineous marriages:

Studies on consanguineous marriages in Pakistan show that up to 50% of the marriages are between close relatives and another 35% take place between Biradri relatives (Bittle 1994). In this study consanguineous marriage was as popular among urban as among the rural families. The practice varied between the individual families i.e. some favouring it more while others did not favour it at all.

Studies on consanguineous marriages are mostly based on a cross-sectional view of the population (Shami and Zahida 1980; Wahab and Ahmad 1996). The present study is unique because it provides an opportunity to study individual families and to retrospectively examine changes in the pattern of marriages with time. The information derived from almost all families clearly indicates that the frequency of consanguineous marriages has increased significantly in the youngest generation of couples as compared to their predecessors (Fig: 9.1b). The changes were similar in the subject (index) as well as control families. This is contrary to the possibility of finding greater numbers of consanguineous couples amongst index families because they formed a selected group with a recessive disorder.

A significant decline in the frequency of consanguineous marriages was observed in the recent generations of Western Europe, North America, and Japan (Coleman 1980; Lebel 1983; Imaizumi 1986). This is attributed to industrialization, greater population mobility, a decline in family size, and higher literacy rates. A similar change would also be expected in other populations. Consanguineous marriage still remains popular without any sign of decline in its frequency in a vast majority of the population of Middle East, Central Asia, North Africa, and the Indian Subcontinent (Bittles 1990). Modell and Kuliev (1992) have anticipated that the absolute number of consanguineous marriages may increase in future because of a high population growth-rate and a fall in the infant mortality leading to survival of greater number of children to reproductive age.

Darr and Modell (1988) noted an actual increase in the 1st cousin marriage in British Pakistanis and it was tentatively attributed to the effect of migration. Wahab and Ahmad (1996) also observed a similar change in Pakistan. The results of this study also suggest that consanguineous marriages have significantly increased in the recent past. The first choice for marriage in Pakistan is mostly confined to a close circle of near relatives or to a wider circle of kinsmen called Biradri. A marriage outside this circle is considered only when a suitable match is not available (Punjabi 1976). An increasing trend in consanguineous marriages observed in the recent generations of the study families, which in fact is a change from marriages within the Biradri to a closer circle of relatives, may

well be due to decreased infant mortality and greater availability of cousins. If this is true then, at least in the immediate future, the trend will increase still further.

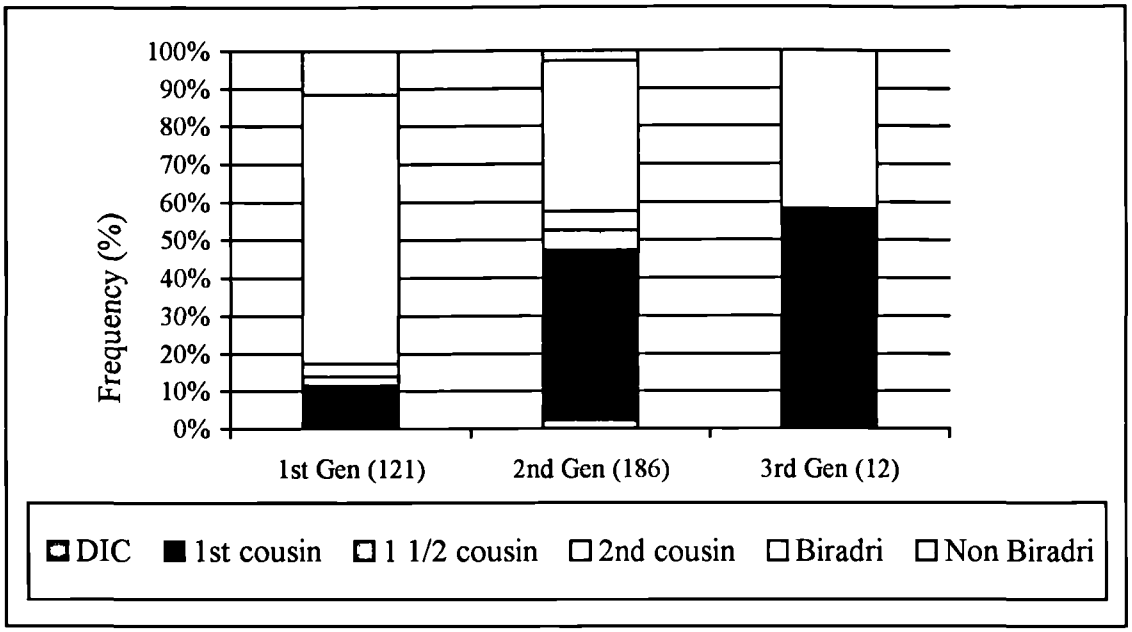


Fig: 9.1b. Pattern of marriages in the three generations of the study families.

No published data are available on the frequency of consanguineous marriages in the previous generations of Pakistan. Before the partition of the Subcontinent the population of present Pakistan was an admixture of Hindus and Muslims. Hindus of Northern India are mostly exogamous (Sinclair 1972) and consanguineous marriage amongst the Muslims of the present Northern India is around 33% (Basu 1975). Contemporary figures from the adjacent part of Punjab in Pakistan show about 50% consanguineous marriages (Shami and Zahida 1980). Compliance of marriage pattern with the local social pressures may cause variation in marriage preferences (Malhotra 1979). The marriage pattern amongst Muslims of Northern India may well have been influenced by a general lack of preference for a similar trend in the Hindu community. After the partition of the Subcontinent the Muslims in Pakistan have been relieved from the social pressure which the Muslims in the Northern India are still experiencing. It is likely that the frequency of consanguineous marriages amongst the Muslims of pre-partition India were not different from the Muslims

of present Northern India. An increasing tendency of consanguineous marriages seen in this study may partly be due to a change in the social circumstances.

The changing pattern of marriages is also reflected in the coefficient of inbreeding for the last three generations of the study families. The average coefficient of inbreeding by generation showed a progressive rise with time (Table: 9.13). The pattern was similar in the subject as well as the control families. For example the value in the youngest generation was 0.0352, which is approximately 25% higher than the estimated 0.0280 for the Pakistani population (Bittles 1994). An extrapolation of the value of F when plotted against time (Fig: 9.2) shows that with the current pattern of consanguineous marriages the value of F by the year 2015 will be something like 0.0575.

Table: 9.13. Coefficient of inbreeding by generation in the study families. A similar pattern was observed in the subject as well as the control families.

Families:	**Coefficient of inbreeding (F):**		
	1st generation	**2nd generation**	**3rd generation**
Subjects:	0.0058	0.0141	0.0343
Controls:	0.0019	0.0105	0.0360
All:	0.0046	0.0128	0.0352

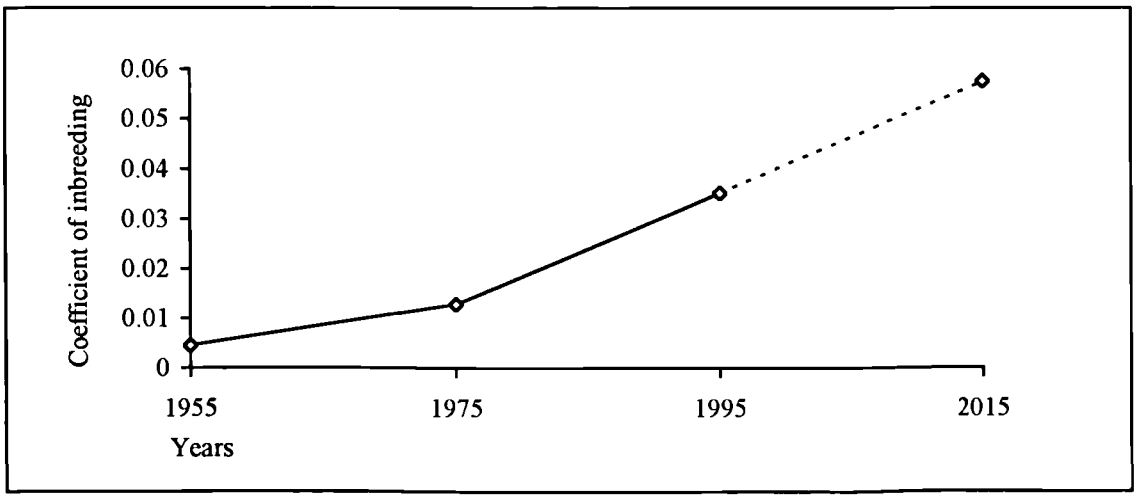


Fig: 9.2. Change in the coefficient of inbreeding over time. Extrapolation of the line to the year 2015 shows that with the current pattern of marriages the F may increase to 0.0575.

Antecedent consanguineous marriages and inbreeding estimates:

The results of this study clearly show that antecedent consanguineous marriages significantly affect the inbreeding estimates. For example the kinship coefficient for a 1st cousin marriage was found to be approximately 15% higher than the theoretically predicted (Table 9.14). Similarly values for the other consanguineous relations were also higher. The results also indicate that the increment in F due to antecedent consanguinity was similar for all degrees of relationship. This suggests that antecedent consanguinity may be more significant in increasing genetic risk for more distantly related couples. Normally consanguineous relations more than 2nd cousins are considered insignificant (Bittles 1994). But this may not be so in Pakistan where distant relatives or even Biradri relatives may be at a significant risk. The results of screening for thalassaemia in the families investigated showed that unrelated Biradri spouses of four carriers had the same thalassaemia mutation. But no carrier was married to another carrier from a different Biradri/Tribe.

A revised calculation of F in different ethnic groups of Pakistan based on the kinship coefficient calculated in this study shows that the predicted value in Punjabis are closer to the mean coefficient of inbreeding seen in the youngest generation of the study families (Table: 9.15).

Table: 9.14. Coefficient of kinship for a consanguineous relationship in the most recent generation of the study families.

Coefficient of kinship (Φ):				
	DIC	**1st cousin**	**1½ cousin**	**2nd cousin**
Expected:	0.1250	0.0625	0.0313	0.0156
Observed:	0.1348	0.0718	0.0330	0.0242
% increase:	8%	15%	5.5%	55%

DIC: Double first cousin

DNA based estimate of inbreeding in a randomly selected sample investigated in this study also showed that the estimates were generally higher than those calculated by the

conventional methods. However, these were significantly lower than the estimates obtained by pedigree analysis. It appears that pedigree analysis may be more suitable for inbreeding estimates. It was also observed that the values obtained for different alleles in the same sample varied considerably. For a more realistic picture it might have been appropriate to use a STR locus with fewer number of alleles or to investigate a larger sample size.

An important factor that is not taken into consideration when making inbreeding estimates is the practice of marriages within a circle of Biradri. Bittles (1994) has shown that up to 85% of the marriages in Pakistan take place within a Biradri circle. In this study an even stronger tendency (94%) of marriages within the Biradri or Tribe was observed. The term Biradri literally means "brotherhood". It is a community of large-scale descendent group whose members are related either closely or distantly by blood or by marriage so that the members think of each other as kinsmen (Kolenda 1978). Family members of the spouses arrange a typical Pakistani marriage. The Biradri or Tribe members are given priority and a marriage outside this circle remains under criticism for the rest of the life of those contracting such a relationship (Punjabi 1976). The Biradri circle can be seen as a relatively isolated population. In one of the families investigated in this study it was observed that three out of four couples who had children affected by thalassaemia major were non- consanguineous but were from the same Biradri. Incidentally all of these couples had the same β-thalassaemia mutation i.e. IVSI-1, a mutation that is seen in 5% of the general population. These findings highlight the genetic consequences that a marriage within a Biradri circle can bring.

The usual methods of calculating inbreeding coefficients, even by drawing extensive pedigrees, would be unable to incorporate the contributions made by Biradri relationships. The use of a polymorphic DNA marker on a large number of unrelated Biradri members may be of help in solving this problem. In the two study families, in whom STR analysis was carried out, only 12 unrelated Biradri members in Family No: 1 and 4 in Family No: 2 were available for DNA analysis. This number was too small to draw conclusions about the inbreeding estimates.

Table: 9.15. Observed and predicted coefficient of inbreeding in the five major ethnic groups of Pakistan.

Ethnic Group (n):	**Proportion of couples and their contribution for the *F* based on the figures observed in this study:**						**Coefficient of inbreeding:**		
	DIC: (0.1348)	**1st Cousin: (0.0718)**	**1½ Cousin: (0.0330)**	**2nd Cousin: (0.0242)**	**Biradri Member:**	**Unrelated:**	**Observed:**	**Predicted:**	**Reference:**
Punjabi (9,520):	0.9% 0.00122	37.1% 0.0266	11.7% 0.00386	0.6% 0.00014	33.9% 0.000	15.8% 0.000	0.0280	0.0318	Bittles 1994
Pathan (2,037):	-	22.0% 0.0158	5.3% 0.00175	6.8% 0.00165	8.3% 0.000	57.6% 0.000	0.0164	0.0192	Wahab and Ahmad 1996
Sindhi (202):	5.0% 0.00674	55.4% 0.0398	5.9% 0.00185	6.9% 0.00167	21.3% 0.000	5.4% 0.000	0.0437	0.0501	Dr. Rafique Memmon Personal communication.
Baluchi (189):	-	84.1% 0.0604	-	2.6% 0.0006	? 0.000	13.2% 0.000	0.0532	0.0610	Dr. Jaleel Anwar personal communication.
Mohajir (120):	0.8% 0.00011	24.2% 0.0174	8.3% 0.00274	14.2% 0.00344	? 0.000	52.5% 0.000	0.0209	0.02095	Mr. Mohammad Iqbal personal communication

DIC: Double 1st cousin

Genetic effects of consanguineous marriage:

Thalassaemia, a recessive disorder whose carriers can be identified by simple blood tests, can be used as an example to investigate the factors that may determine the number of at risk couples for a recessive disorder in families where marriages are predominantly consanguineous. In this study two main factors were identified that correlate with the number of at risk couples. These include (1) the number of carriers in a generation and (2) the coefficient of kinship (Φ) for the generation. In most families at least 30% carriers were present in a generation where at risk couple was seen.

The situation in Family No: 8 was interesting because the thalassaemia gene was first introduced in this family by an unrelated Biradri member from the 1st generation (Fig: 9.10). In the 2nd and 3rd generations the number of carriers had increased but it was not enough to allow marriage between two carriers. If the current pattern of marriages continues and no efforts are made to avoid marriages between carriers, it is expected that over the next 2-3 generations a couple at risk might be seen. This suggests that in the presence of about 45% consanguineous marriages it may take 4-5 generations before a recessive gene can manifest itself. Spontaneous recessive mutations may also take a similar number of generations to manifest in such families.

Most of the at risk couples were present in the 2nd generation of the index families. In the same generation approximately 30% of the unmarried individuals were also carriers. If a thalassaemia carrier in Pakistan chooses to marry a close family relative then there is a theoretical possibility that in about 30% his or her spouse will also be a carrier as compared to only 5% if he or she marries an unrelated person. The observed risk of marrying a close cousin was slightly lower than expected which may be due to the small numbers investigated. It was observed that 25% of the carriers were married to a close cousin and in about 22% of the carriers married to a cousin the spouse was also a carrier. In contrast, none of the carriers was married to a carrier from another Biradri/Tribe. In 25% of the carriers married to a Biradri/Tribe member the spouses were also carriers. The actual risk of thalassaemia in a consanguineous marriage in Pakistan would be approximately four times higher than a completely unrelated marriage. The risk in a

consanguineous marriage for the rare recessives, that collectively may be more common than thalassaemia, would be even more marked (Modell and Kuliev 1992).

The relationship between kinship coefficient and the number of at risk couples in a generation for the common consanguineous relations is straightforward. It is more complicated for Biradri/Tribe members. Normally inbreeding estimates do not take into consideration the contribution made by a Biradri or a Tribe member. The data from this study suggest that, at least for a common recessive disorder, a marriage within the Biradri or a Tribe circle may also be at a higher risk.

DNA polymorphism data from the two study families indicate that genetic drift, due to a small number of the family members, may also contribute towards fluctuation in the carrier rate and the number of at risk couples. Fig: 9.3 shows how random drift can influence gene frequencies between generations. The allele frequency can vary from one generation to another and some alleles may change significantly to a higher or a lower level within the span of relatively few generations. A typical Pakistani family can be seen as a small population isolate. The size of this isolate is maintained and partitioned from the rest of the population due to frequent consanguineous marriages. Consanguineous marriage in it self does not affect gene frequencies (Modell and Kuliev 1992). However, it may act indirectly by maintaining the finite size of the population. In a small population group like this, drift may help to cause fluctuation in the frequency of a particular gene (Bodmer and Cavalli-Sforza 1976).

In the five control families, 4/56 (7%) of the consanguineous couples had deaths of children due to causes that were possibly genetic in origin. In contrast, none of the 68 non-consanguineous couples from these families had deaths of children due to genetic causes (p=0.03). However, there is only circumstantial evidence that the children of the consanguineous couples had a genetic disorder. The studies on association of consanguineous marriages and increased mortality carried out in less developed regions face a major problem in diagnosing cause of death and under such circumstances it is difficult to partition mortality into genetic and non genetic components with any confidence (Bittles 1994). Interaction between consanguinity and social variables can also

complicate to a significant extent assessment of the genetic effects of consanguinity. Consanguineous marriage is more likely to be favoured by the poorest and least educated families, whose children are at greatest risk of elevated rates of infant and childhood mortality from the effects of infections and nutritional deficiencies. Failure to control for socio-economic differentials can readily lead to biased estimates of the adverse effects of consanguinity (Bittles 1995). A recent study from Pakistan shows that the frequency of both consanguinity and birth defects were related with the socio-economic levels of the study groups, but there was no association between inbreeding and birth defects (Yaqoob et al, 1993). The apparent lack of association, when correction for other confounding factors is applied, may be due to elimination of the deleterious recessive genes over time from a community where consanguineous marriages are customary for several generations (Sanghvi 1966). The deleterious recessives, however, cannot be completely eliminated because of new mutations, reproductive compensation and heterozygote advantage (Bittles 1980). Studies on consanguinity and genetic disorders indicate that the average mortality in the progeny of a 1st cousin marriage is 4.4% higher than an unrelated marriage. The excess mortality is due to the presence of about 1.4 recessive genes per person that are lethal in the homozygous state (Bittles and Neel 1994). The results from this study support the later observations because the affected and the unaffected couples from the families, especially the controls, were living under identical socio-economic conditions that largely rules out the possibility of involvement of confounding variables.

The subject families were a selected group because of a history of recessive disorder in the family whereas the controls were randomly selected families. A comparison between the number of affected couples in the two groups showed that in the subjects there were 11/79 (14%) consanguineous couples who had children affected by a genetic disorder and in the controls there were 4/56 (7.1%) couples with affected children. The difference between the two groups was statistically insignificant (p=0.26).

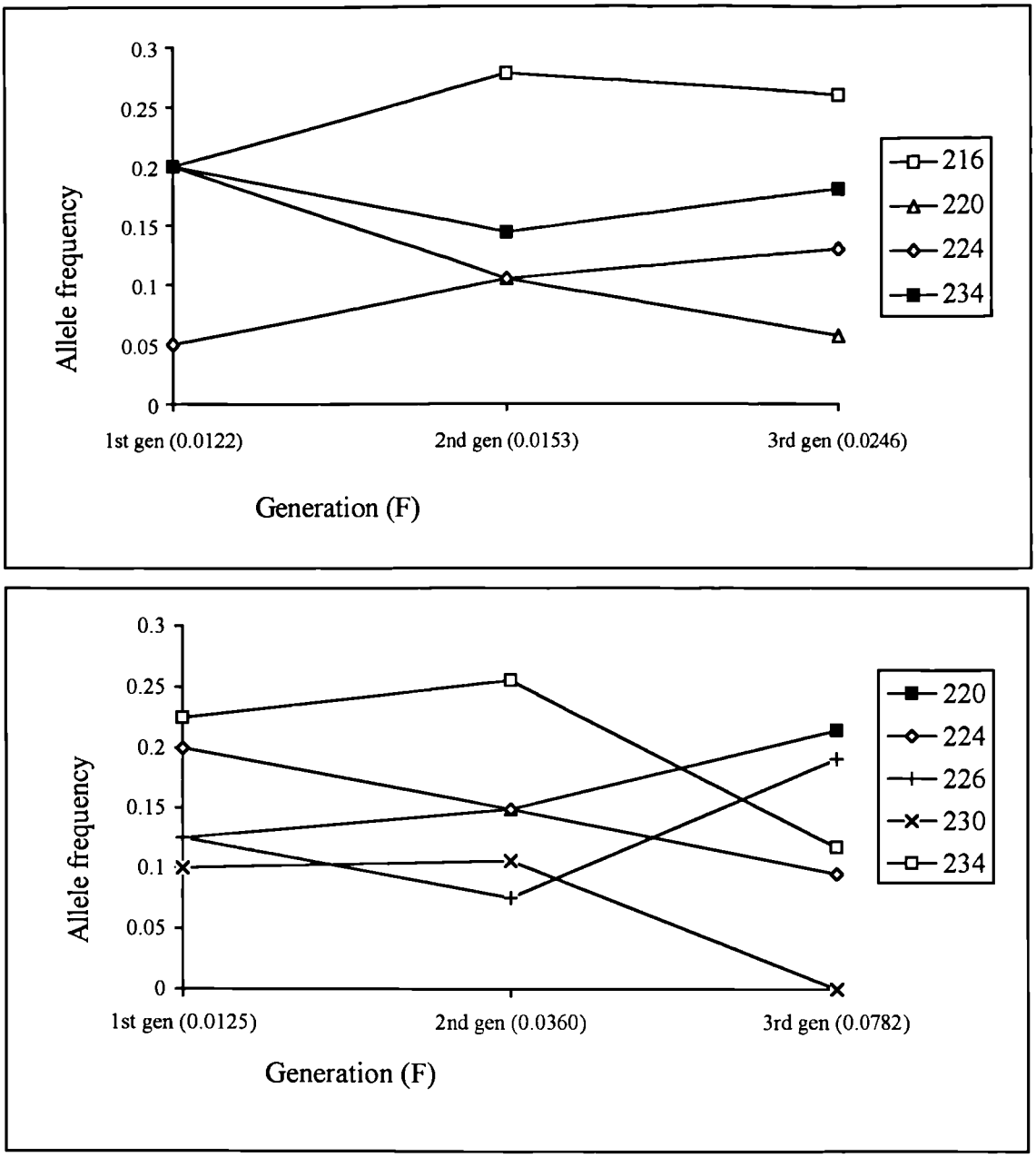


Fig: 9.3. Variation in D21S11 allele frequencies in the three generations of Family No: 1 (top) and Family No: 2 (bottom).

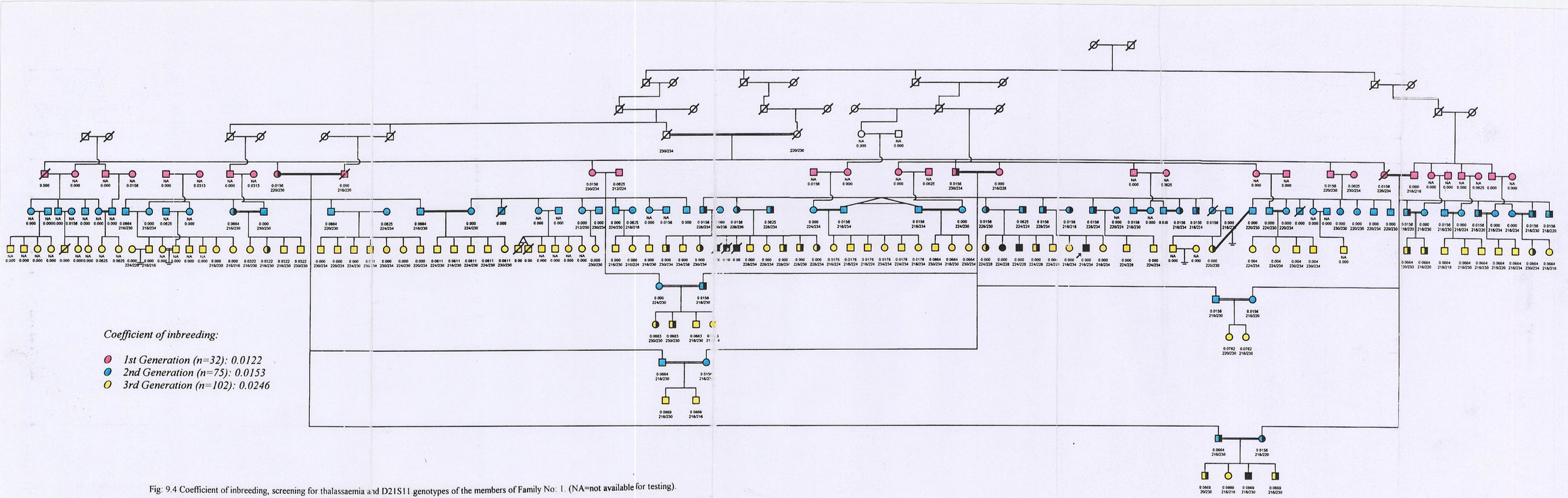


Fig: 9.4 Coefficient of inbreeding, screening for thalassaemia and D21S11 genotypes of the members of Family No: 1. (NA=not available for testing).

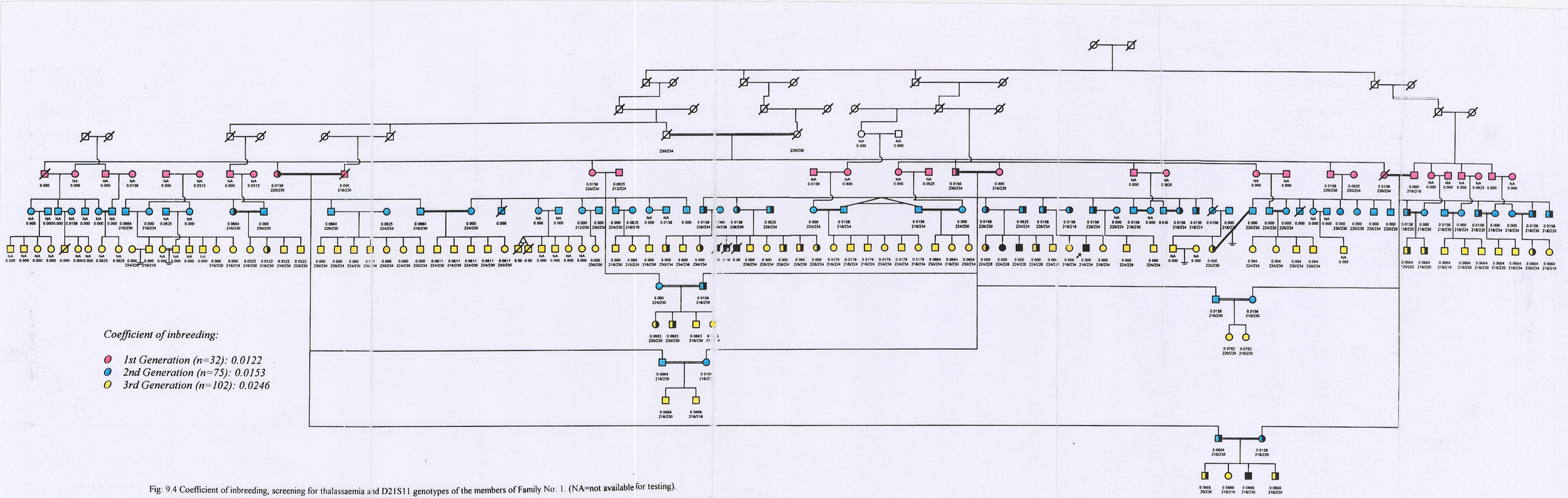


Fig: 9.4 Coefficient of inbreeding, screening for thalassaemia and D21S11 genotypes of the members of Family No: 1. (NA=not available for testing).

Fig: 9.5 Coefficients of inbreeding, screening for thalassaemia and D21S11 genotypes of the members of Family No: 2. (NA=not avaliable for testing).

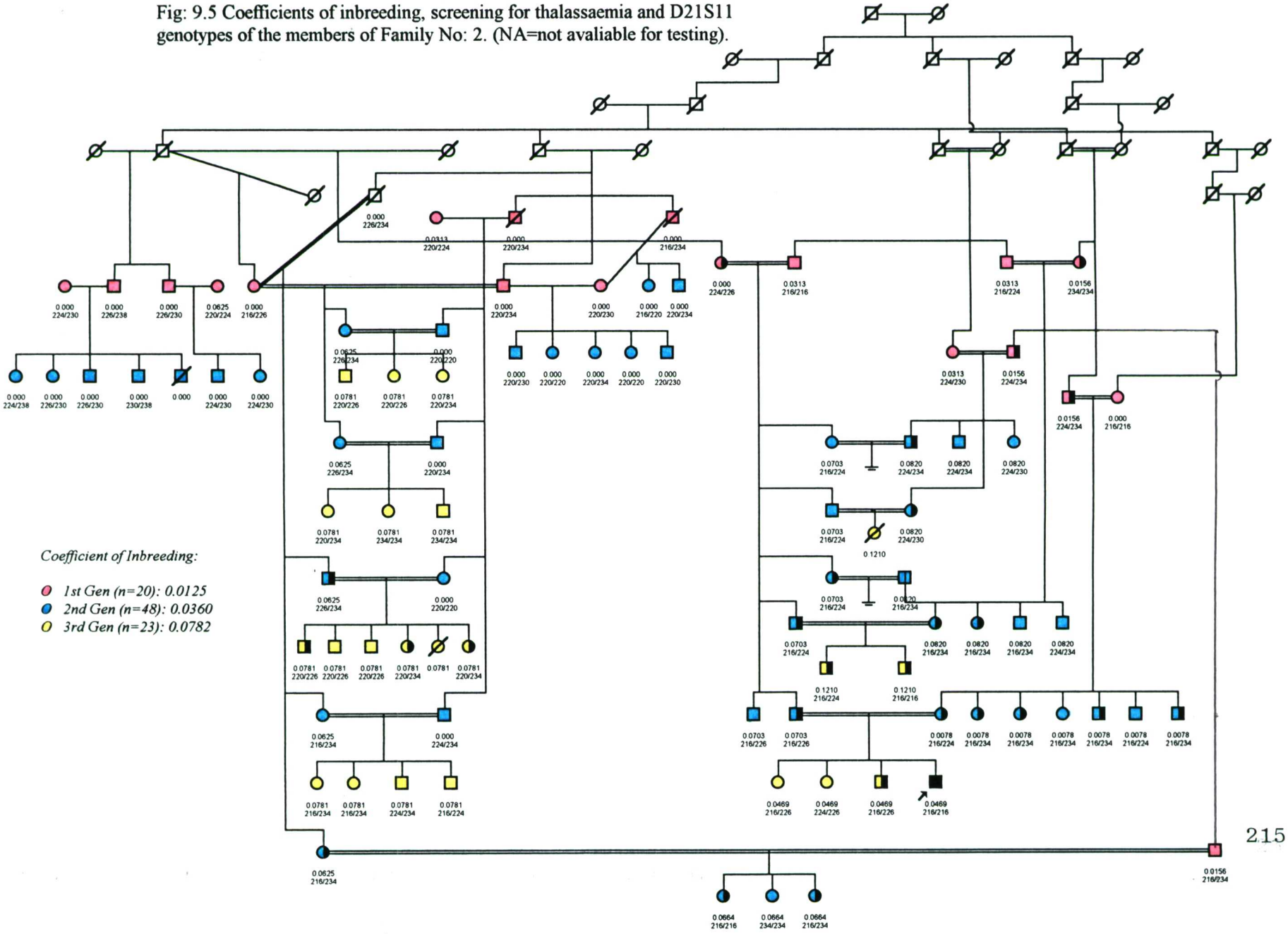


Fig 9.6. Coefficients of inbreeding and thalassaemia screening in the members of Family No: 3. (NA = not available for testing).

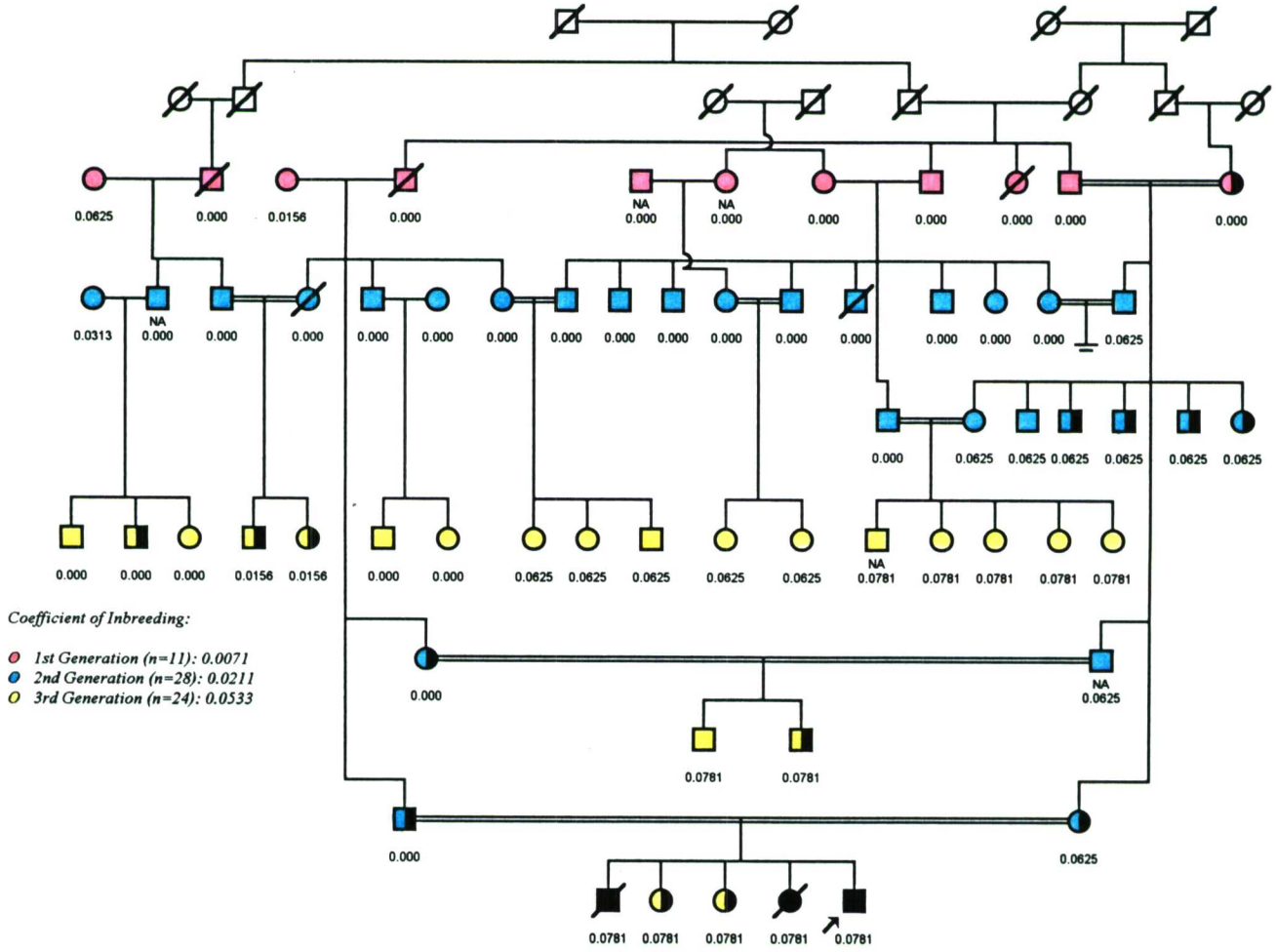


Fig: 9.7. Coefficient of inbreeding and thalassaemia screening in the members of Family No: 4. (NA=not available for testing).

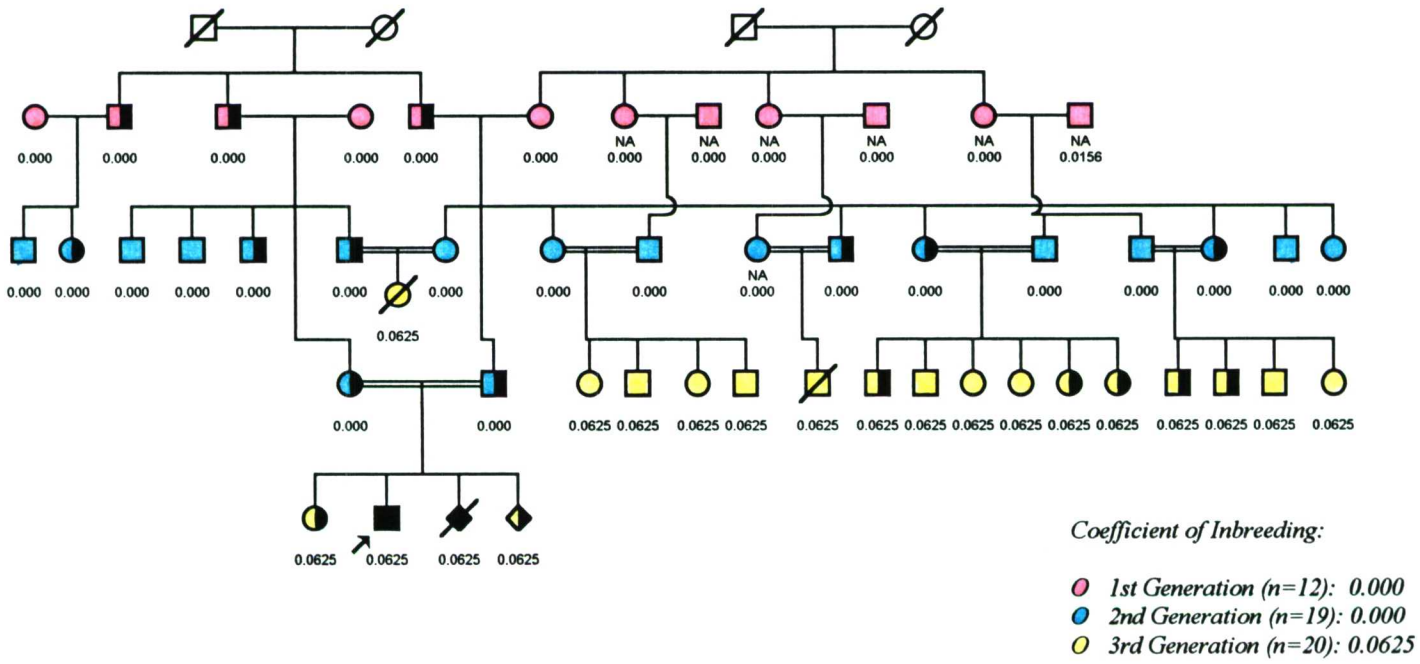
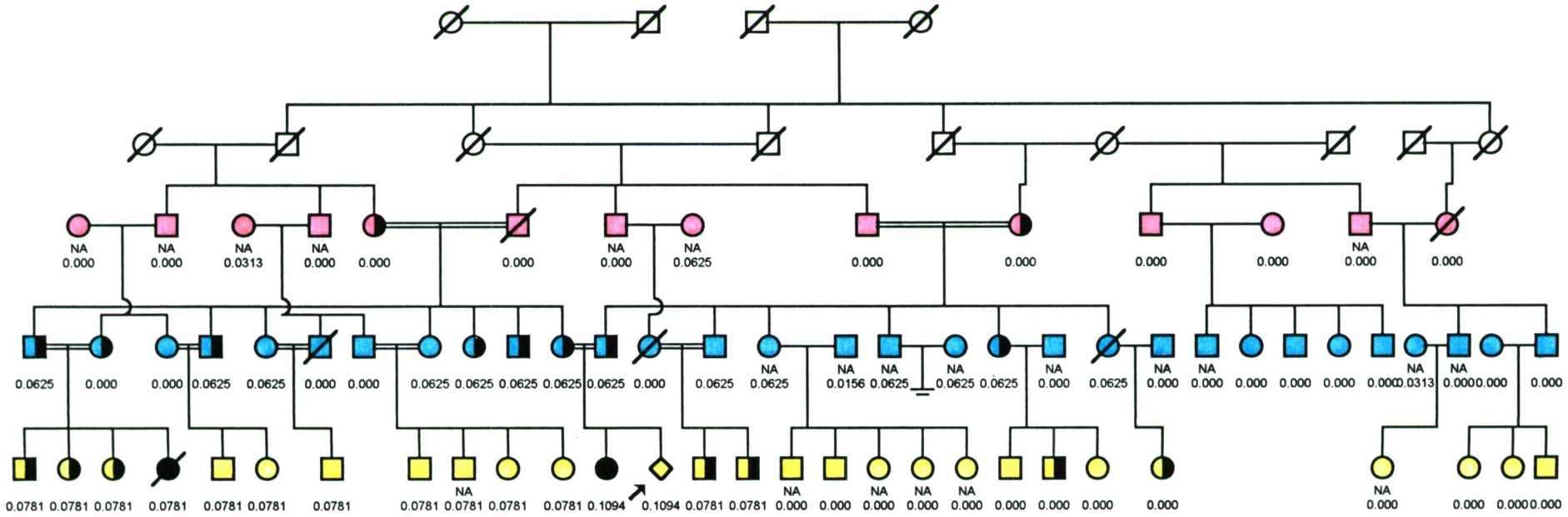


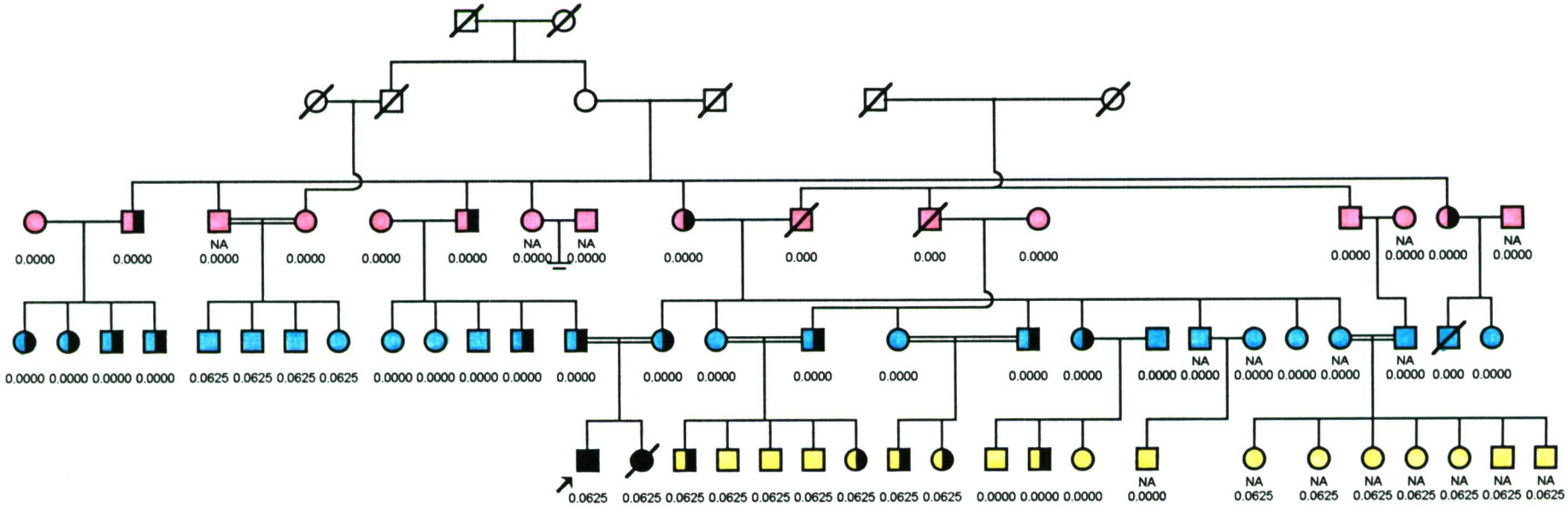
Fig: 9.8. Coefficient of inbreeding and thalassaemia screening in the members of Family No: 5. (NA=not available for testing).



Coefficient of Inbreeding:

- *1st Generation (n=14): 0.0067*
- *2nd Generation (n=31): 0.0297*
- *3rd Generation (n=28): 0.0441*

Fig: 9.9. Coefficients of inbreeding and thalassaemia screening in the members of Family No: 6. (NA=not available for testing).



Coefficient of Inbreeding:

- *1st Generation (n=16): 0.000*
- *2nd Generation (n=27): 0.0093*
- *3rd Generation (n=20): 0.0500*

Fig: 9.10. Coefficient of inbreeding and thalassaemia screening in the members of Family No: 8. (NA=not available for testing).

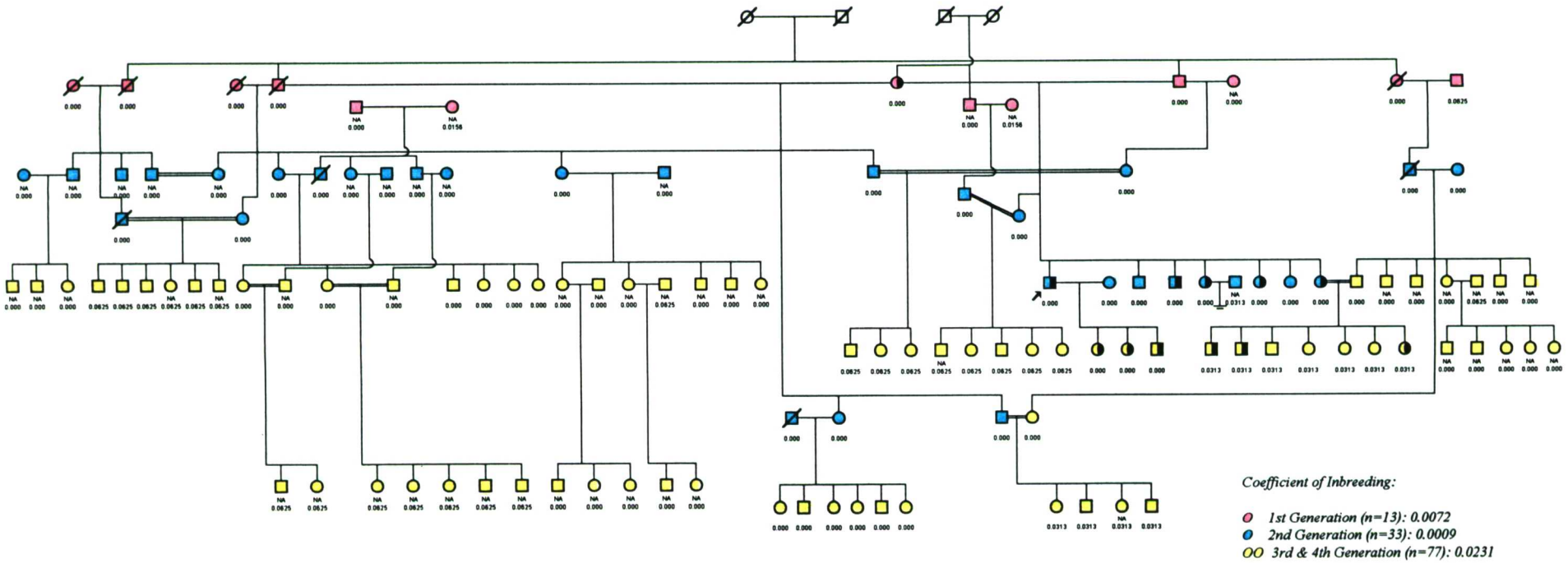
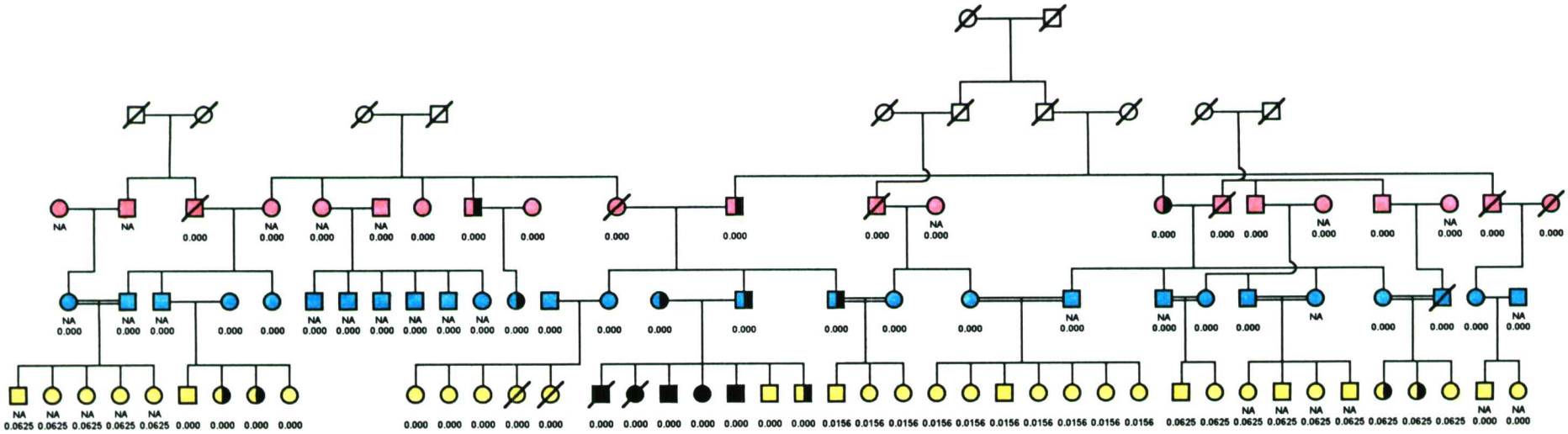


Fig: 9.11. Coefficient of inbreeding and screening for sickle cell disorder in the members of Family No: 9. (NA=not available for testing).



Coefficient of inbreeding:

- *1st Generation (n=21): 0.000*
- *2nd Generation (n=28): 0.000*
- *3rd Generation (n=42): 0.0245*

Fig: 9.12. Coefficients of Inbreeding and screening for sickle cell disorder in the members of Family No: 10. (NA=not available for testing).

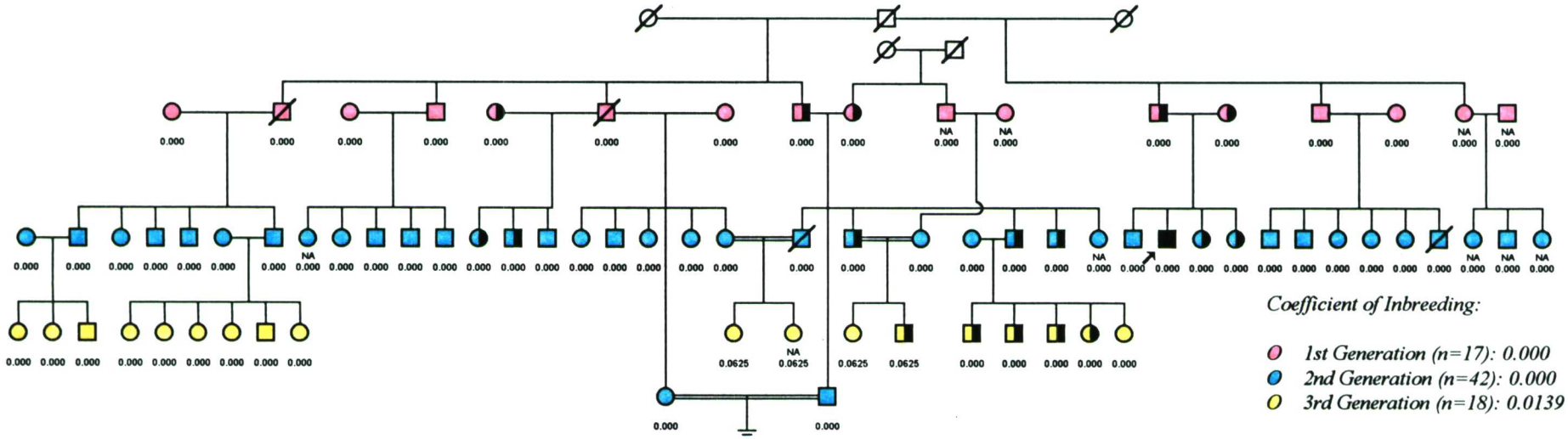


Fig: 9.13. Coefficient of inbreeding and screening for thalassaemia in the members of Family No: 11. (NA=not available for testing).

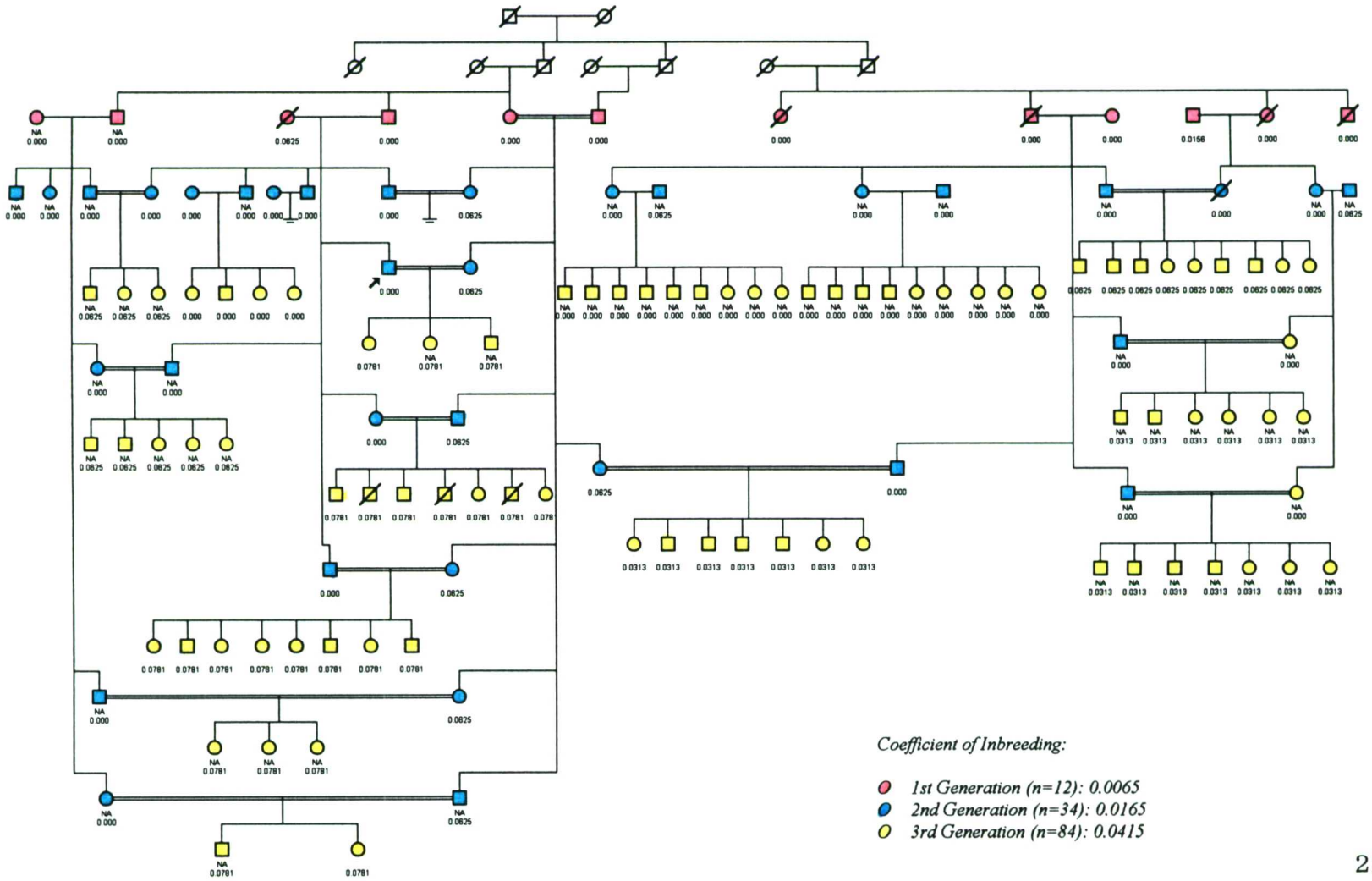


Fig: 9.1 4 Coefficient of inbreeding and thalassaemia screening in the members of Family No: 12. (NA=not available for testing).

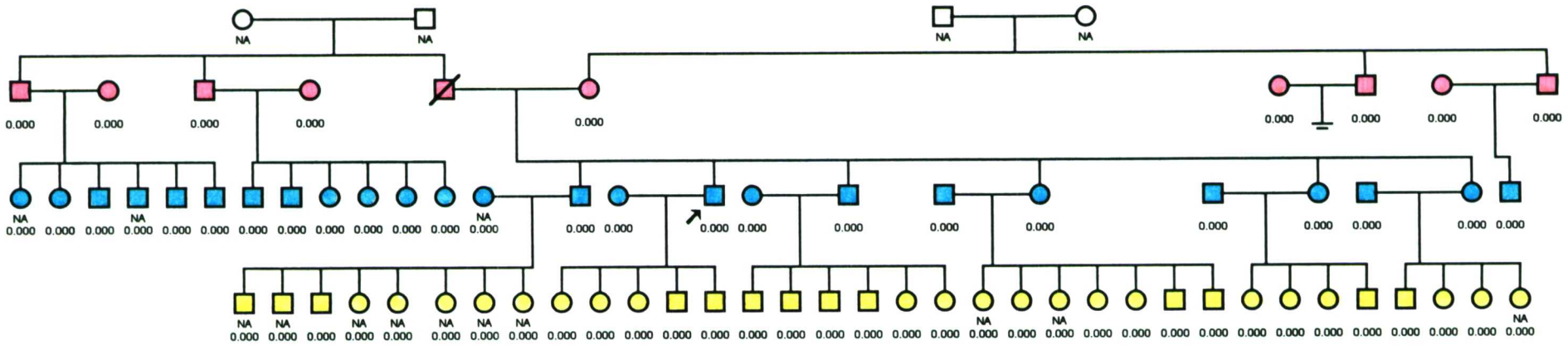


Fig: 9.15. Coefficient of inbreeding and screening for thalassaemia in the members of Family No: 13. (NA=not available for testing).

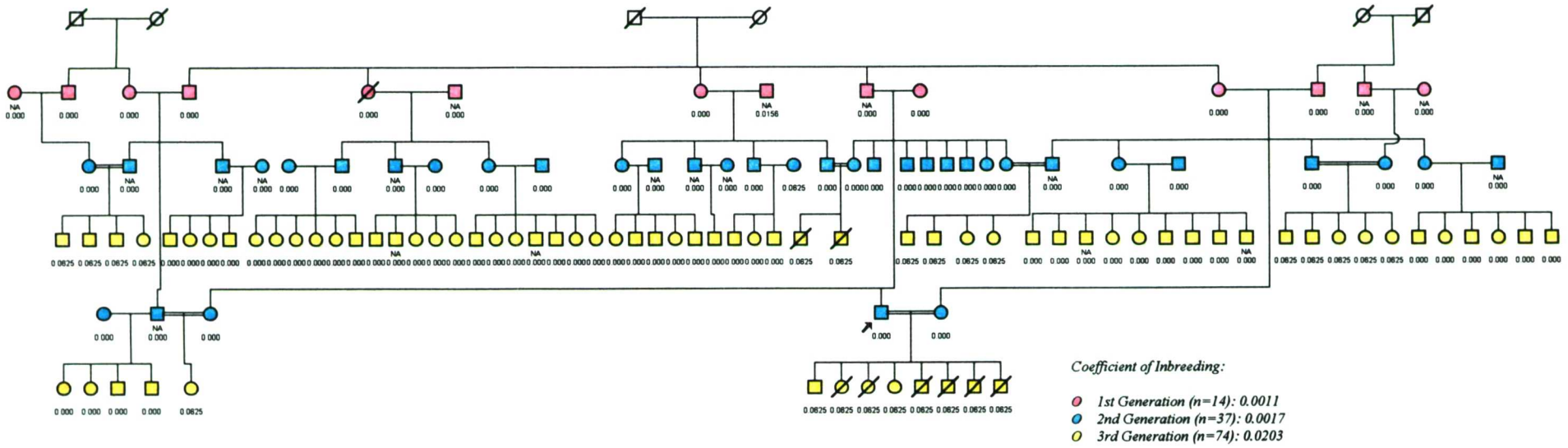


Fig: 9.16 Coefficient of inbreeding and screening for thalassaemia in the members of Family No: 14. (NA=not available for testing).

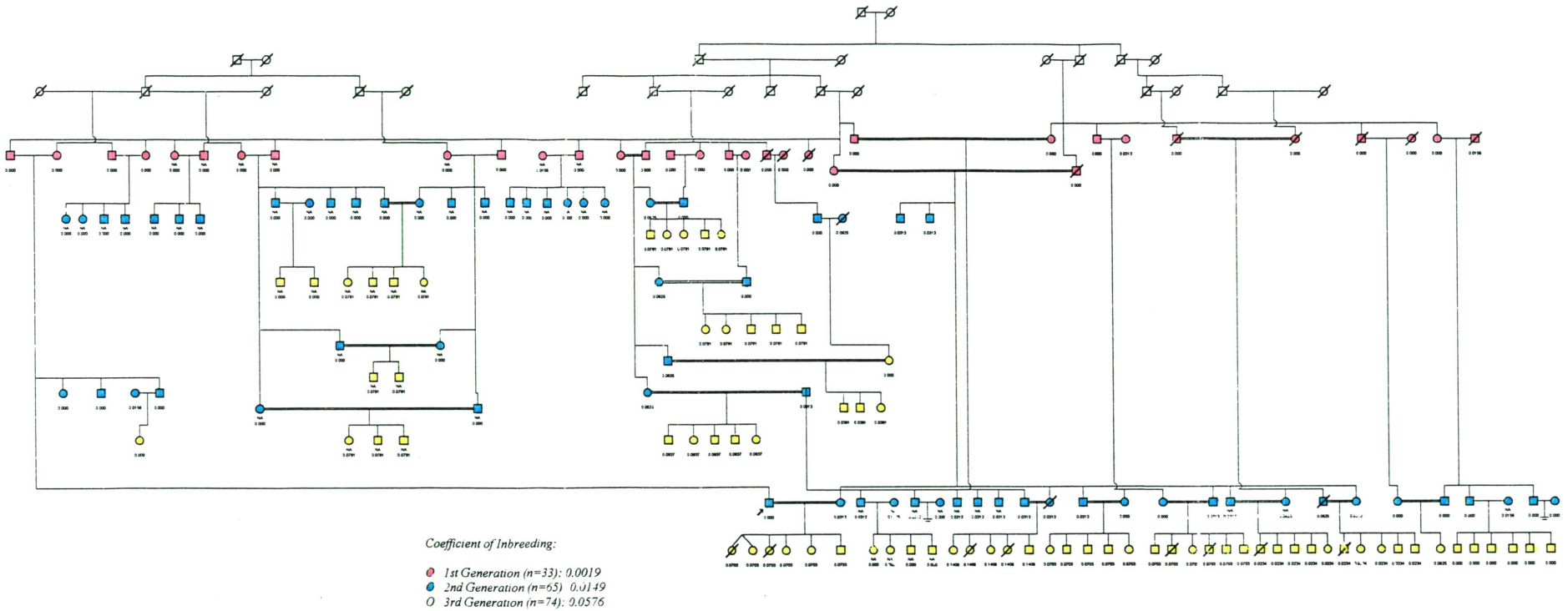
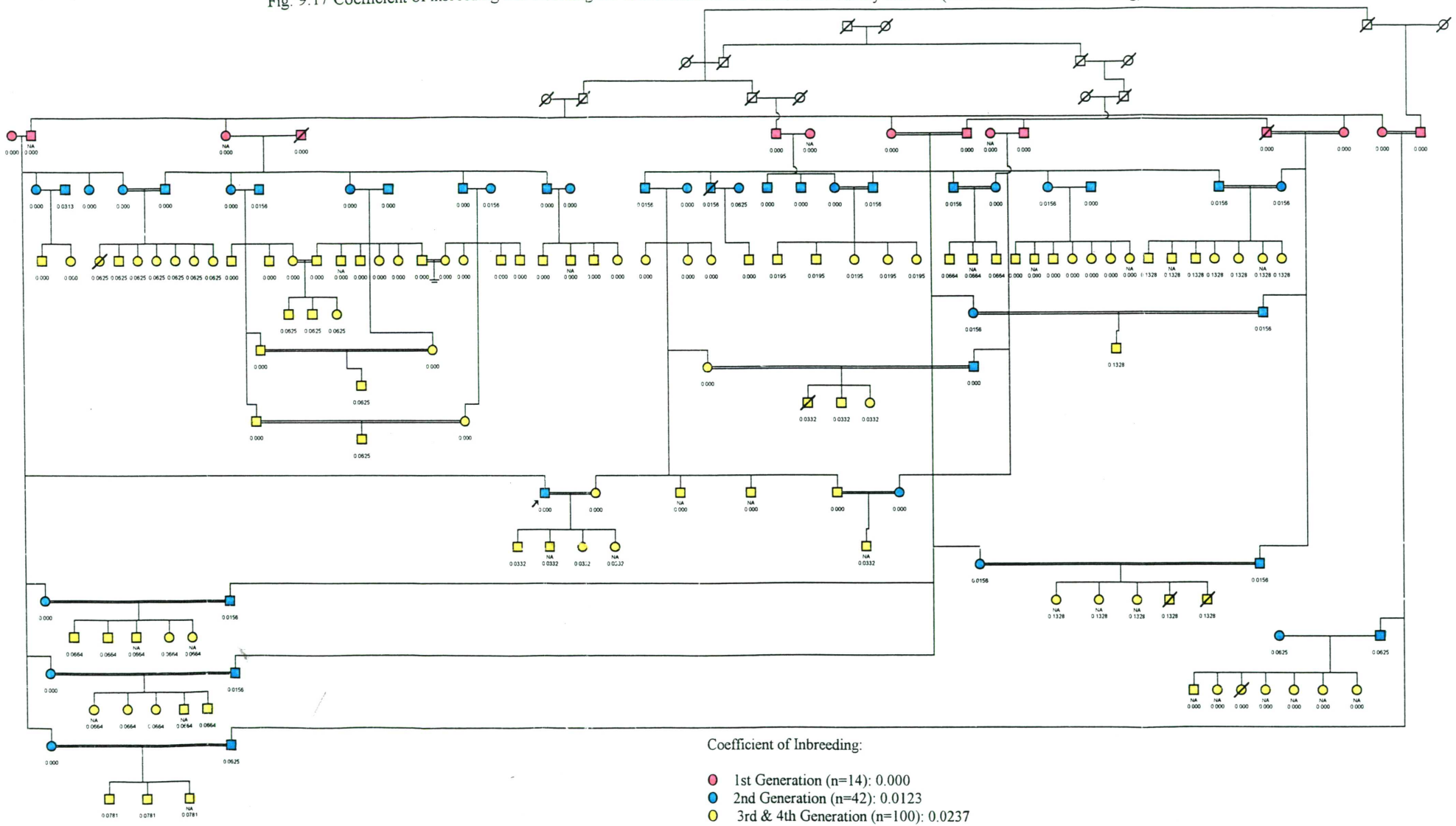


Fig: 9.17 Coefficient of inbreeding and screening for thalassaemia in the members of Family No: 15. (NA=not available for testing).



Coefficient of Inbreeding:

- 1st Generation (n=14): 0.000
- 2nd Generation (n=42): 0.0123
- 3rd & 4th Generation (n=100): 0.0237

10 Final discussion

Introduction:

There is a long standing experience of preventive programmes at the national level in many Mediterranean countries. This is illustrated in the Cyprus thalassaemia prevention programme that has been highly cost effective and has resulted in almost complete control of thalassaemia. In principle, a programme on similar lines could be applied in any country where haemoglobin disorders are common (Alwan and Modell 1997). This would involve improvement of treatment facilities through adoption of a common management protocol, awareness and involvement of the community, population screening and counselling, and availability of prenatal diagnosis. The success of the Cyprus programme is attributed to low infant mortality, small size and small population, high standard of living and health care, high literacy and low birth rate, existence of a national health infrastructure and Government's involvement (Angastiniotis et al, 1986).

Haemoglobin disorders in Pakistan are of sufficient public health importance to justify a disease-oriented control programme (WHO 1993). However, development of a prevention programme on the lines of a Mediterranean programme is largely precluded at present in a developing country like Pakistan by a large population, high birth rate, low rate of literacy, limited resources, and lack of organization of health services. Another issue of fundamental importance is the attitude of a Muslim community towards various options for prevention.

At least five "policies" for thalassaemia exist in different countries of the Eastern Mediterranean Region (EMR) including Pakistan (Alwan and Modell 1997). These include:

1. No treatment, counselling or prevention.

2. Best possible patient care, plus retrospective genetic counselling after the birth of an affected child.
3. Same as 2 plus the option for prenatal diagnosis in subsequent pregnancies.
4. Same as 2 plus prospective screening and counselling but no prenatal diagnosis.
5. Same as 2 plus prospective screening, genetic counselling with prenatal diagnosis.

An ideal "policy" for thalassaemia should include measures to provide adequate treatment facilities, genetic counselling and prospective identification of at risk couples before they give birth to an affected child. Until recently, the "policy" of no treatment, counselling or prevention was widely prevalent in the EMR including Pakistan. At present in many countries of the region treatment facilities are more widely available and retrospective counselling is also being provided to many families. Prenatal diagnosis is available to only a limited number of the families. The response of most of the affected families to prenatal diagnosis is therefore mostly unknown. The ideal "policy" for Pakistan is a long way in the future because of the general low level of development in the country and the limited resources available. Therefore, a basic question at present is where and how to start?

Keeping in view the infant mortality of around 90/1000 in Pakistan (Burney 1993) the Government's priority is to reduce it with basic programmes of primary health care, immunization and nutrition etc. (Annual report of health services in Pakistan 1995-96). Effective implementation of such programmes has caused a very rapid fall in infant mortality in many countries. As a result chronic disorders including thalassaemia very rapidly became more important and even in some countries the health priorities also changed e.g. Iran, Maldives and Thailand. This tends to happen when infant mortality falls to below 40/1000 because (a) more vulnerable children survive (b) they become conspicuous, get diagnosed and their management can be very costly (Prof B. Modell personal communication). Pakistan is far away from a situation where mortality due to genetic disorders will become conspicuous. But the problem is important and will emerge in due course of time (Fig: 10.1). Therefore, some form of planning must begin now.

The population of Pakistan is very diverse because a small urban section with better education is more developed as compared to a large rural and mostly uneducated section

(Fig: 10.2). Most thalassaemic children are diagnosed and get treated in the developed section of the population whereas affected children born in the less developed section usually die undiagnosed and parents often replace them with healthy children. A demographic transition would occur when people have better education and fewer children who survive. With this the problem which is emergent only in the developed section would also become prominent in rest of the population. Since the problem is only emerging the Government is unlikely to become interested at present. The important questions at present are:

What level of Government interest should realistically be promoted at present?

It should include:

a. An awareness of the problem and its scale and the awareness that it will become a major problem when the present health policies succeed.
b. Support (at least moral) for research and appropriate approaches for treatment and prevention of thalassaemia in Pakistan.
c. Encouragement for formation of nuclei and centres from which appropriate services can spread in due course when needed.
d. Willingness to have a dialogue with the NGOs and others to ensure progress.
e. Ensuring that the programme enters into planning at Government level.

How to achieve political will?

The usual route for achievement of political will is through increasing the Government interest in the problem (Fig 10.3). National experts, leading clinicians and thalassaemia support associations of the parents and patients can positively influence the Government authorities (Alwan and Modell 1997). The availability of epidemiological data collected through reliable sources can be effective in presenting the problem to the health authorities. The NGOs could write a report on the state of thalassaemia in Pakistan every year and send it to the ministry of health. An increasing number of patients who would survive with the availability of treatment facilities can also attract Government's attention. Collaboration of all interested parties is a key element. As long as different people tell the Government different things they will never listen.

Pakistan at present is in the earliest stage of developing a programme for thalassaemia. The work carried out in this pilot study is essential for forward planning. The results have brought forward guidelines to formulate a realistic future policy for thalassaemia in Pakistan.

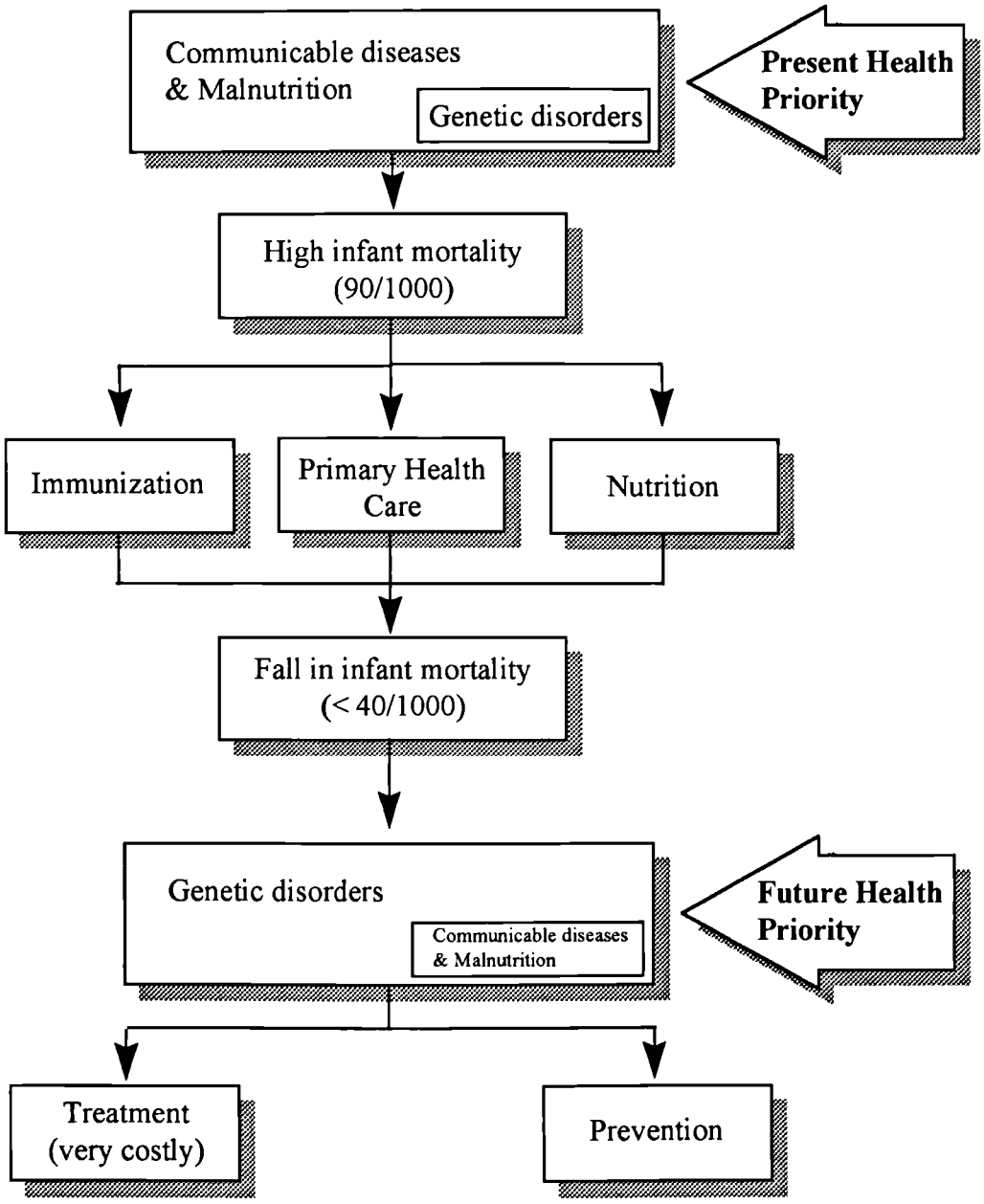


Fig: 10.1. The usual route of creating Government's interest in genetic disorders. The present health priority of the Government is to reduce the infant mortality by preventive health policies. With the success of these measures, genetic disorders will emerge as an important health priority. Therefore some planning must begin now.

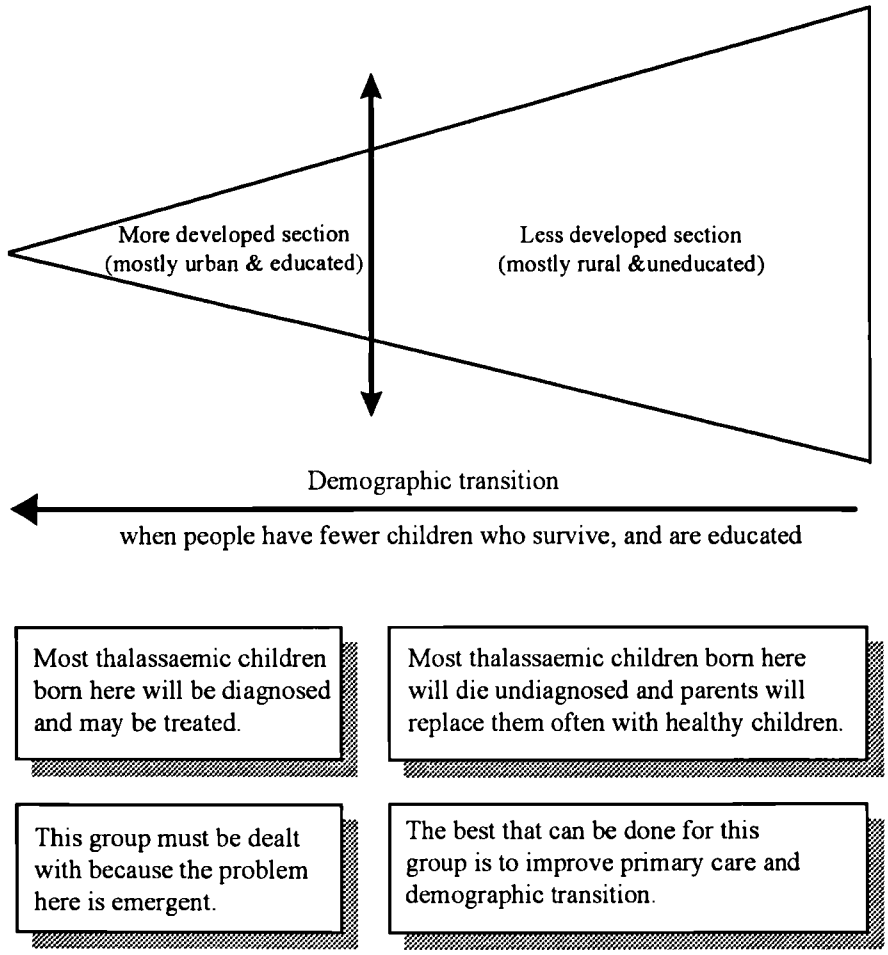


Fig: 10.2. The population of Pakistan is diverse which has a small more developed and a large under developed section. At present most thalassaemics are seen in a smaller section of the population that is mostly urban and has better education. With demographic transition the problem is likely to become more and more obvious (Prof. B. Modell personal communication).

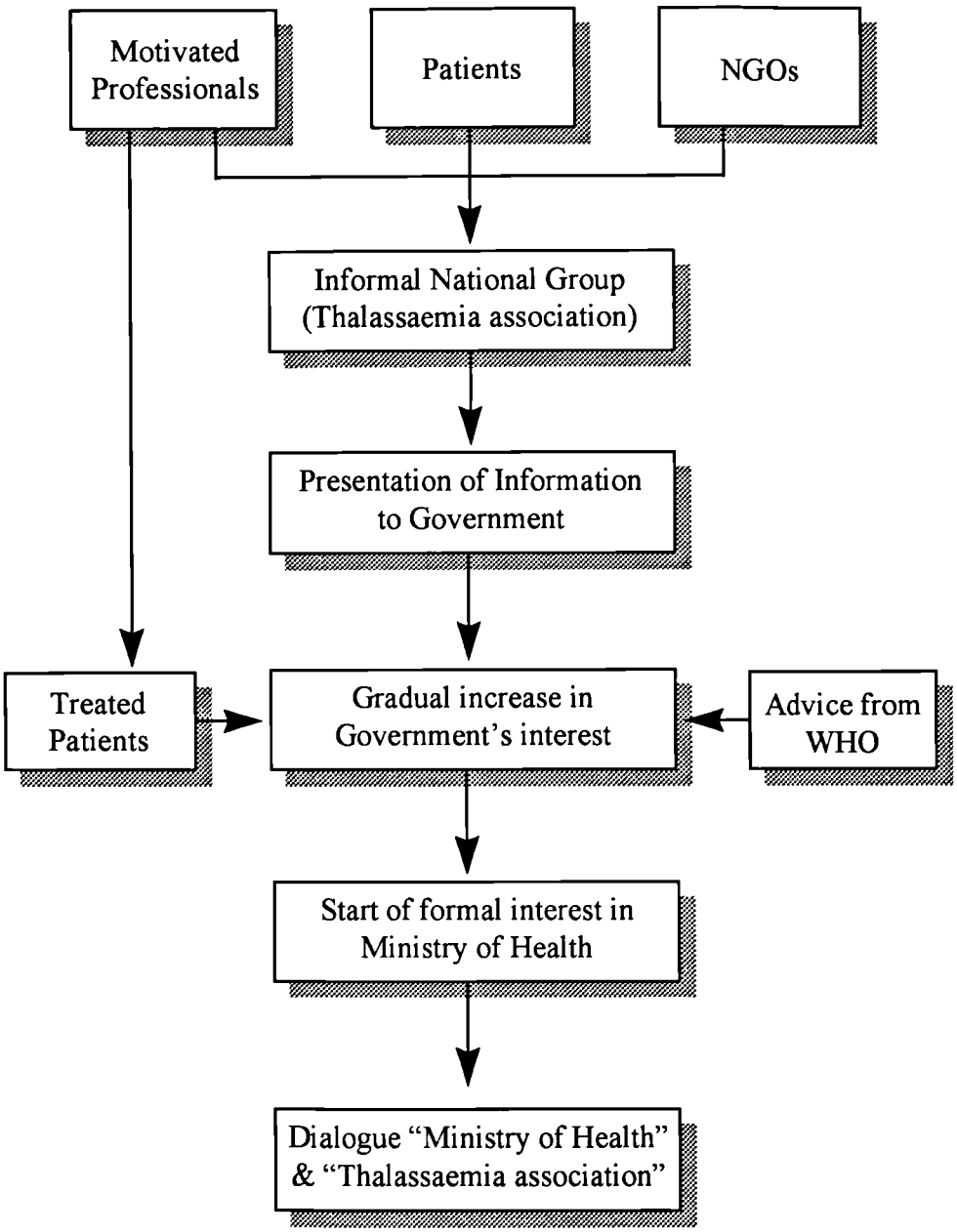


Fig: 10.3. The usual route for achievement of political will. Pakistan at present is in the earliest stage of developing a programme for thalassaemia (Prof. B. Modell personal communication).

Epidemiology of haemoglobin disorders in Pakistan:

WHO (1985) has estimated that the carrier rate for β-thalassaemia in Pakistan is around 5%. The study of haemoglobin disorders by Khattak and Saleem (1992a) gave the first indication that the carrier rates for β-thalassaemia may be different in various ethnic groups. It is important to investigate carrier rate in the ethnic groups because β-thalassaemia genes have an uneven distribution. Moreover, the frequency of consanguineous marriage is also different in the ethnic groups of Pakistan (Bittles 1994; Wahab and Ahmad 1996). This would affect the calculation of annual birth rate of thalassaemia major in each group. The overall carrier rate for β-thalassaemia in this study was found to be 5.3% (95% confidence limits: 4.1-6.5%). The carrier rate also varied in the ethnic groups (Table: 10.1). The abnormal haemoglobins could not be screened in this study. However, other published reports on abnormal haemoglobins indicate that they are not significant as compared to β-thalassaemia. The largest study of 5000 Pakistani individuals by Sharma et al, (1976) showed that 0.78% had an abnormal haemoglobin including Hb-D (0.42%), Hb-E (0.18%), and Hb-S (0.18%).

The carrier rate of thalassaemia is used to calculate the annual number of births of affected children. This information is essential for planning a national programme for control and prevention of the disorder (Alwan and Modell 1997). The estimated annual number of thalassaemia major births in Pakistan, based on the carrier rate and the frequency of consanguineous marriages in each ethnic group, would be approximately 4550 (1.2/1000 new births) (Table: 10.1). Maximum affected births are expected in Punjabis (1.12/1000) which also is the largest ethnic group (80 million). The highest birth rate, however, is expected in Baluchis (4.3/1000). This is due to a high carrier rate combined with a high frequency of consanguineous marriage. The affected birth rate amongst Sindhis (0.324/1000) is low because of a low carrier rate (1.3%) in this group.

The annual births of children with an abnormal haemoglobin, based on the figures of Sharma et al, (1976), are estimated at 99 new cases each of homozygous Hb-S and Hb-E. An additional 181 cases each of Hb-S/β-thalassaemia and Hb-E/β-thalassaemia are also expected every year. Therefore the overall annual number of children born with a

clinically significant haemoglobin disorder is estimated at 5116 (approximately 1.35 per 1000 births) and its major portion (89%) is represented by β-thalassaemia. The total number of pregnancies at risk would be approximately 20500. The estimated total number of births of children affected with a clinically significant haemoglobin disorder and the number of at risk pregnancies in Pakistan (Fig: 2.2 and 2.6) is the highest in the Eastern Mediterranean Region (Alwan and Modell 1997).

Table: 10.1. Carrier rates for β-thalassaemia and the estimated number of births with thalassaemia major in the five major ethnic groups of Pakistan.

Ethnic group:	**Population:**	**Carrier rate:**	***F*:**	**Estimated births/1000:**	**Annual cases:**
Punjabi	80 million	4.5%	0.0280	1.12	2454
Pathan	20 million	5.2%	0.0164	2.09	610
Sindhi	15 million	1.3%	0.0437	0.32	136
Baluchi	7 million	9.0%	0.0532	4.31	844
Mohajir	15 million	5.2%	0.0209	1.21	506
Total:	137 million	5.3%	0.0287	1.35	4550

Awareness campaign:

An essential prerequisite of a thalassaemia prevention programme would be to educate the public as well as the health professionals about the basic concept of thalassaemia prevention. The adult literacy rate in Pakistan is 35% and the rates for the urban and rural areas are 58% and 28% respectively (Economic survey 1995-96). In a predominantly illiterate community special means of educating the public would be required. This study shows that treating physicians are the most important source of information for the affected families. Therefore, the first and the most important aspect of educating the population would be to educate the doctors. At present very little genetics is taught in the undergraduate medical colleges in Pakistan (Hassan et al, 1997). Even at the postgraduate level genetics is a neglected speciality. Physicians, nurses, and social workers in practice need to be informed of new technologies and approaches. Special courses in genetics may

be arranged. Medical genetics newsletter may be a good idea for the busy medical professionals where selected topics may be regularly discussed. Education for the young population can be achieved through education in schools. It is also worth noting that because of the young age structure of the population, education efforts focused on schools can have a relatively greater impact than in most developed societies (Alwan and Modell 1997). Regular programmes about thalassaemia on radio and television can also be useful.

A simple and effective first step would be to focus on informing the families reporting at the treatment centres. Experience has shown that most of the affected families have very little information about the disease and they readily lend their ears to any advise that could be useful for them. The use of television for disseminating information on thalassaemia was quite effective. Easy to understand booklets on thalassaemia, although not as effective as television due to low rate of literacy, were also useful. The experience of the couples who have used prenatal diagnosis was also an important medium of information for other families.

Provision of improved treatment facilities:

The affected parents are unlikely to become attracted to prevention unless they are assured about the health of the sick child. An essential step before prevention can be offered, would be to improve the treatment facilities. Since thalassaemia is not the Government's health priority at present the treatment is mostly left to Non-Governmental Organizations (NGOs) who are looking after over 7500 registered thalassaemics in the country. Unfortunately, there is no uniformity in management protocols at the centres run by the NGOs. There is also no audit on the quality of treatment being offered (Saleem 1996). Till such time that thalassaemia becomes Government's health priority the NGOs would be required to improve the standard of treatment at their centres. It can be improved substantially by agreement amongst the health professionals involved, adoption of a common management protocol, and their audit at frequent intervals (Angastiniotis et al, 1986).

National co-ordination committee for thalassaemia:

The National co-ordination committee may be required when the importance of thalassaemia is recognized. This may be a long time in the future. Since the problem is emergent some planning must start now. Competent leadership and effective co-ordination between various governmental and non- governmental organizations is essential. A national co-ordination committee is required for planning, monitoring, and evaluation of the progress of the work. The responsibilities of the committee should be to focus on formulation of policies, preparation of a national plan and mechanisms for co-ordination. The committee should include a programme co-ordinator, specialists in medical genetics, haematology, paediatrics, community medicine, public health and health education. The programme co-ordinator should be a highly motivated and knowledgeable person. He or she should either be based in the Ministry of health or work in co-ordination with the responsible health officers (Alwan and Modell 1997).

Carrier screening for haemoglobin disorders:

Carrier screening has played a major role in thalassaemia prevention programmes in the Mediterranean (Cao 1987). The Mediterranean experience clearly demonstrates the value of incorporating a community based control programme into the primary health care system to ensure effective delivery to the population (Angastiniotis et al, 1986). Since the primary health care system is not well developed in Pakistan (Burney 1993) thalassaemia prevention through this would be unrealistic. Screening of the whole population is also not reasonable due to administrative and financial constraints. A common approach for identifying at risk couples in Europe is the screening in pregnancy (Cao 1987). A major limitation of this approach in a developing country like Pakistan is that the vast majority of the pregnant women, especially in the rural areas, cannot be screened because they do not report to an antenatal clinic. According to a survey, trained personnel attend only 26% of pregnant women in Pakistan (Burney 1993). Under these circumstances it is very unlikely for antenatal screening to be practically feasible. The approach may be suitable for countries like the UK where most women attend antenatal care in early pregnancy (Modell and Berdoukas 1984). Another important aspect of screening during pregnancy is the gestation at which the risk is discovered. If it is discovered late it is unlikely that it

would lead to abortion even if the fetus were affected.

Clearly, an alternative approach for Pakistan is needed. It may be appropriate to have a selective approach for (a) more developed part of the population within reach of the services (b) families who already know about thalassaemia. The idea of targeting a selective population of index families is attractive (Alwan and Modell 1997). However, there are no reports on the success of this approach. In this pilot study, that is the first of its kind in which the families affected by thalassaemia were targeted, many points of interest were highlighted. The study is not big enough to lead to a real conclusion as yet and further research e.g. long-term follow-up of the families is required.

Identification of at risk couples:

One of the objectives of thalassaemia screening is the identification of at risk couples. The majority of thalassaemic couples in Pakistan are identified retrospectively when they already have one or more affected children. In communities where family size is small retrospective identification of the couples and offering them prenatal diagnosis is unlikely to reduce the incidence of new births of thalassaemia major (Cao 1987). By contrast, when the final family size is large, retrospective counselling may lead to either cessation of reproduction or prenatal diagnosis. This can reduce the affected birth rate in the community by up to 50% (Alwan and Modell 1997). However, further reduction in the birth rate of the affected children would require prospective identification of at risk couples. In this study the alternative approaches of screening in pregnancy and screening the index families were investigated.

Antenatal screening:

In this study screening of 350 pregnant women could not identify any at risk couple as compared to a theoretical possibility of 1 in 200. This could be due to the small numbers studied. But there is a clear indication that screening of a large number of individuals would be required to identify only a few at risk couples. Another difficulty may arise due to late identification of at risk couples when prenatal diagnosis or termination of pregnancy may not be acceptable to the couples. Only 18% of the pregnant women screened were in the first trimester. The late discovery of risk may also produce dramatic

emotional stress on the families (Petrou et al, 1990).

Screening in the index families:

The results of screening in several small to large families show that the approach is technically feasible and acceptable to almost 2/3rd of the families. Screening the index families on an average identified 31% carriers per family. By contrast, there was not a single carrier amongst 397 members of the five families without a known history of a haemoglobin disorder. Screening in a similar number from the general population would have identified at least 20 (5%) carriers. This suggests that very frequent marriages between close relatives or Biradri/Tribe members "traps" normal as well as abnormal genes within the family. These results strongly support that screening in the affected families would be the most rewarding in this setting. Interestingly, the carrier rate amongst consanguineous Pakistani families is not higher compared to a non-consanguineous Cypriot family with history of thalassaemia (Mouzouras et al, 1980). This is because approximately 50% of the 1st degree relatives of a carrier would be carriers whether the marriage pattern is consanguineous or non-consanguineous. Consanguineous marriage as such would have little effect on the carrier rate of an abnormal recessive gene in the family. However, in a family with predominant consanguineous marriages the key factor is the general high risk of relatives who may marry each other.

There were approximately 29% unmarried carriers in a family. If all marriages in a family are consanguineous then at 29% carrier rate 8.4% of the carriers would be married to another carrier. The frequency of consanguineous marriages in these families was around 45%. At this rate 13% (29% of 45%) of the carriers would be married to 13% of the related carriers while the remaining 16% of the carriers would be married to 5% of the unrelated carriers in the general population. This would mean a total of 2.5% (1.7% plus 0.8%) of the couples in a family would be at risk. On an average there were 22.5 couples per family. It is estimated that 2.5% of the 22.5 couples (0.56 couples per family) would be at risk. The observed number of at risk couples per family, however, was higher (1.7 per family). The difference is because the study families are selected and all but one had at least one affected child. In the family without an affected child the index person was a thalassaemia carrier. It means that all families that are targeted because of a history of

haemoglobin disorder may not have at risk couples. This may be especially true when the index person is a carrier rather than an affected child.

Effect of screening on the marriage choices:

The main objective of population screening for thalassaemia is the identification of carriers early enough for marriage choices to be affected. Experience from a research study in Greece shows that premarital screening has had very little effect on the choice of partner (Gosden 1993). Studies from Cyprus also show that discouraging marriages between carriers had proved unacceptable and was abandoned (Wald et al, 1988). The follow-up for one year after screening in the index families studied shows that there was some impact on the marriage choices. However, this is too short a period to draw firm conclusion and clearly further research on this subject is needed. Preliminary results suggest that premarital screening and preventing marriages between carriers may present itself as an acceptable approach in a Pakistani setting. This may be particularly important for the families who do not accept prenatal diagnosis on religious ethical or cultural grounds. Premarital screening may be expected to have a long term benefit if the carriers are suitably informed. Premarital screening is being considered in a number of countries of the Eastern Mediterranean Region and has already been mandated in Iran (Alwan and Modell 1997).

Other advantages of screening the index families:

There are several other advantages in screening the index families. For example, the screening can be initiated by counselling of one or a few individuals in a family. The rest of the family members are likely to follow the guidance of the leading members. A critical step would be to identify the right person as the starting point. The key person in a family can be identified by asking others to whom they go for advice. Some of the families who refused the offer for screening mostly did so because of the lack of a key person. The social set up in most Pakistani families, particularly in the rural areas, is such that a few family elders handle most of the important affairs (Punjabi 1976). The obedience of the younger generations is considered obligatory. Therefore it would be appropriate to involve the family elders in the screening exercise. Marriage in a Pakistani set up is mostly planned by women, but the final decision whether to accept or to reject the proposal may

lie with the men. While carrying out screening inclusion of both men and women would be essential.

There also are technical advantages in carrying out a targeted screening. For example it can be started from the eldest available members. The children may be screened only when one or both of their parents are found to be carriers. A practical problem may arise, because in a field trip to a rural area it may be difficult to screen the elders first and sample the children at a later stage. One tube osmotic fragility test (Kattamis et al, 1981) can be very useful for guiding on the spot whether to include or exclude a person from screening. This may substantially reduce the number of individuals requiring screening.

Genetic counselling:

At present most of the couples who seek genetic counselling in the Eastern Mediterranean Region, including Pakistan, are already married and have one or more affected children (Alwan and Modell 1997). Once the genetic diagnosis is made the couples learn that the treatment of the child is complex and life long and there are chances of recurrence of the disorder in future pregnancies. A choice to have no further children may be relatively simple for couples who have several healthy children. The choice may be difficult for the young couples. The study shows that prenatal diagnosis is technically feasible and is also accepted by the majority of the retrospectively identified couples. This may reduce stress within the families by broadening choices away from focus on choice of partner. It therefore would reduce stigma, stress and fear. Continuing and sustained efforts are needed for counselling the affected couples who report to the treatment centres. This is not only important for the couple itself but it also provides an opportunity to access a large number of other family relatives who may be at risk. Alwan and Modell (1997) have suggested that retrospective counselling of the affected couples where the final family size is large may reduce the birth incidence of thalassaemia major to almost 50%. The offer of prenatal diagnosis to such couples can further reduce the birth rate of affected children. The response of the prospectively identified at risk couples to genetic counselling could not be assessed in this study as this is a long term undertaking. Clearly more research is needed to investigate this aspect.

Genetic counselling aims at replacing misunderstandings with correct information about the cause of genetic disease and resources available for its diagnosis, treatment and prevention. It is usually done according to the internationally accepted guidelines (Harper 1993). The approach however may be modified according to the local cultural, social and religious beliefs (Alwan and Modell 1997). The issues that need consideration while formulating an approach for counselling Pakistani families include (1) illiteracy (2) confidentiality (3) stigmatisation and (4) consanguineous marriages.

Illiteracy:

Comprehension of the genetic concepts correlates with the level of education (Yuen et al, 1988). However, even in well educated communities the effect of counselling on the knowledge of carrier state and its implications may be small (Barrai and Vullo 1980b). The situation may be worse in a predominantly uneducated community as in Pakistan where it may be difficult to grasp even the basic concept of a genetic disorder. The aim of providing an informed choice can be achieved only if the parents understand the implications of the choices. Therefore the genetic counsellor in addition to providing information and giving an informed choice, may have to help in making the right choice. The counsellor should not be over-directive or the individuals may feel guilty about their choice. Counselling may be made more effective by using visual aids and illustrated booklets.

Counselling would be easier if the person has already seen affected children. While screening the index families most members would have at least some knowledge about the affected child's illness. This is a significant advantage when the literacy rate is very low. It would be unrealistic to expect an illiterate Pakistani lay person to understand or believe they carry a very severe disease, which they have never seen or heard about previously. Counselling therefore would be easier and more effective when delivered to members of a thalassaemic family.

Confidentiality:

Confidentiality of results would be a key factor in counselling of individuals in a family. During screening in the families it was observed that some individuals had reservations

about screening because they thought it might cause difficulties in arranging marriages of their children. The marriage in a traditional Pakistani family is arranged by agreement between the parents of the spouses. The parents try their best to find a match within the family or the Biradri (Punjabi 1976). Therefore if the carrier status of a boy or a girl was known within the family, then these individuals may be rejected as spouses. In one of the families, the mother of a thalassaemic child, who herself was under considerable stress due to the illness of her child, preferred to hide the carrier status of her younger brother while arranging his marriage with another close relative. The targeted screening is attractive but its actual impact on making decisions about marriage preferences in a family may be complicated. In cases where rejection of a carrier is feared, prenatal diagnosis may work as an acceptable solution. Rejection of heterozygotes does not happen in Cyprus because the population is educated and prenatal diagnosis is freely available (Prof. B. Modell personal communication).

Stigma:

Thalassaemia carrier status may be taken as a stigma for the affected person or families. The counselling of carriers should also include efforts to reduce the element of stigmatisation. It may help to inform the carriers of the benefits associated with the disease. The knowledge that the carriers have a natural resistance to falciparum malaria (Weatherall et al, 1989) or ischaemic heart disease (Crowley et al, 1987; Gallerani et al, 1991; Wang and Schilling 1995) may be helpful to reduce stigmatisation.

Consanguineous marriage and implications for counselling:

In populations where consanguineous marriage is customary, an understanding of its genetic implications is essential for developing appropriate genetic services. Numerous studies provide supporting evidence that consanguineous marriage is associated with increased risk of genetic disorders (Bittles 1980). However, the data supports some harm but clearly shows it is far less than generally believed. It is important to understand that the outcome of consanguineous marriage should not be assessed solely in terms of the restricted horizon of medical audit (Bittles 1995). There are numerous social and economic benefits associated with this practice (Alwan and Modell 1997). Our current state of knowledge tends to be overly focused on the undesirable clinical outcome of close

kin marriage, which affect only a minority of families and individuals (Panter-Brick 1991). There is a much larger proportion of couples whose children do not show identifiable deleterious biological effects and to whom the social and economic benefits of a consanguineous marriage appear obvious and natural (Bittles 1995).

This leaves a dilemma for the medical geneticists and others, whether to discourage the practice on medical grounds or not. Attempts to discourage the practice on genetic grounds alone can do more harm than good (Modell and Kuliev 1992). A more appropriate and acceptable solution would be the establishment of educational programmes and to provide counselling and carrier screening if available for the families who already have a child with a recognizable genetic disorder or where there is an unfavourable family history. In Pakistan the progress in developing screening programmes for genetic disorders may be slow because of the limited resources available. A strategy to identify and diagnose genetic risk may be required, that can be gradually developed over time. Haemoglobin disorders are a good group to pilot and develop the approach.

Prenatal diagnosis:

Molecular genetics of haemoglobin disorders:

Plenty of data are available on thalassaemia mutations in people from the Indian subcontinent (Kazazian et al, 1990). But most of the studies have been done on selected individuals settled in the Western countries. Varawalla et al (1991a; 1991b) have studied β-thalassaemia mutations in British Pakistanis from the northern parts of Pakistan. But this study is inadequate for epidemiological purposes because of a small sample size and lack of representation of all ethnic groups. The present study of over 1200 mutant alleles provides a comprehensive picture of β-thalassaemia mutations in the major ethnic groups of Pakistan.

A total of 19 different mutations were identified. The five common mutations, IVSI-5 (G-C) (37%), Fr 8-9 (+G) (25%), del 619bp (7%), Fr 41-42 (-TTCT) (7%), and IVSI-1 (G-T) (5%) account for 81% of the alleles. In each ethnic group four or five common mutations account for over 80% of the alleles (Fig: 10.4).

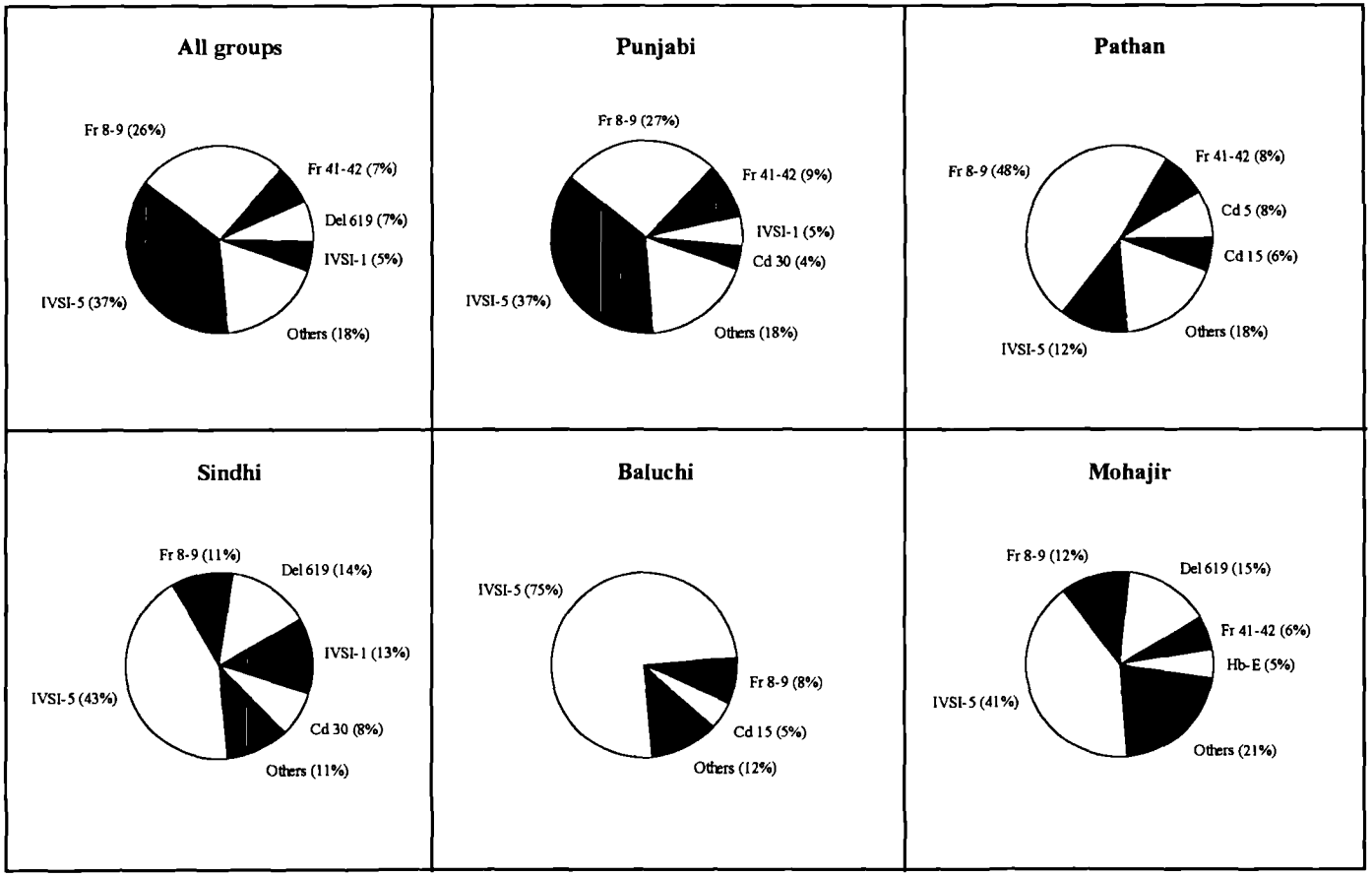


Fig: 10.4. Spectrum of thalassaemia mutations in the five major ethnic groups of Pakistan.

The spectrum of mutations is heterogenous in all of the ethnic groups except in Baluchis where IVSI-5 alone comprises 75% of the alleles. Several factors e.g. the time since appearance of a mutation, population migrations, and random genetic drift might have contributed to the genetic heterogeneity.

Technical feasibility of prenatal diagnosis:

Prenatal diagnosis by direct mutation analysis was possible in over 98% of the couples. In the remaining couples linkage analysis can be used as a backup support. The method is quick, reliable and cost effective. Mutation analysis by multiplex ARMS PCR was also technically feasible and very effective in reducing the cost.

Response of the families to prenatal diagnosis:

There is very little information about the reaction of Muslim families in general and Pakistanis in particular to prenatal diagnosis. Initial information for British Pakistanis suggested a low uptake rate of prenatal diagnosis (Modell et al, 1981). Recent results in the UK show that late referrals, inadequate counselling and a preconception amongst health professionals that Pakistanis do not terminate pregnancies because of religious reasons are the main reasons for low utilization (Petrou 1994). The data from British Pakistanis cannot be extrapolated to Pakistan as British Pakistanis mostly represent people from the Mirpur district in the north of Pakistan that migrated to the UK in the sixties and maintain limited contact with the people back home.

The vast majority of the affected couples in this study (96%) were in favour of prenatal diagnosis and said they would request it in the future pregnancies. Only 56% of the families who had a pregnancy when prenatal testing was available actually used prenatal diagnosis. This finding is not unusual because many people may not actually do what they might have thought of doing in the future (Alwan and Modell 1997). The behaviour of the couples was related to their educational level and socio-economic status. For example, in a randomly selected sample 27% of the couples said they would request prenatal diagnosis in future pregnancies only if the test were free of cost because they could not afford it otherwise. This was probably an important factor in the lower than expected uptake of the test which costs Rs. 3000 ($75). The families may not have been

worried about the cost of treating a thalassaemic child as this was free of charge (blood transfusions only).

This study clearly shows that prenatal diagnosis is accepted by most thalassaemic families in Pakistan. There are two important issues that may determine the long term outcome of prenatal diagnosis and need further discussion. These are (1) termination of pregnancy for a serious genetic disorder and (2) cost of prenatal diagnosis.

Termination of pregnancy for serious genetic disorders:

Islam is the leading religion of Pakistan and it is the faith of about 97 percent of the people. A vast majority of the population follows the Islamic rituals. The low rates of adult literacy in Pakistan make it difficult for the people to distinguish between their religious, traditional and cultural beliefs. This can easily lead to misconceptions about the permissibility or prohibition of anything in religion. In the context of prenatal diagnosis of genetic disorders the most important question is whether termination of pregnancy is permissible in Islam, if the fetus is affected by a serious genetic disorder?

Induced abortion has always been frowned upon in all human societies. As early as the dawn of medical history abortion was taboo. Its proscription was included in the Hippocratic oath and therefore it has been part of the professional heritage from generation to generation up to our own times. The semblance of unanimity on prohibition of abortion however excludes cases where a continuing pregnancy can be harmful for the mother's well being or when the fetus is seriously deformed or defective (Hathout 1974). The views of Islamic jurisprudence on the subject are unanimous in prohibiting abortion if it is carried out on the embryo after animation unless there is a valid reason or excuse justifying it. Concerning abortion before animation the views differ and range from permissibility and disfavour to prohibition (Makdur 1974). The concept of animation is explained in a Quranic verse as follows:

> *"Then We made the sperm into a clot of congealed blood; then of that clot We made a lump (fetus); then We made out of that lump bones and clothed the bones with flesh; then We developed out of it another*

creature" (Al-Quran, Sura Al-Momenoon verse 14).

There is consensus of opinion that "development of another creature" means breathing of soul in to the inanimate fetus (Shafi 1980). However, a difference of opinion is found in the timing of animation. Some people think it coincides with the start of fetal heartbeat (40 days). Most Islamic scholars consider 120 days of embryonic age as the timing of animation, which is based on the following Hadith:

> *"Each one of you is put together in the womb of his mother for forty days, and then turns into a clot for an equal period (of forty days) and turns into a piece of flesh for a similar period (of forty days) and then the soul is breathed into him" (Abdullah, Sahih Al-Bukhari, 8.593).*

The community would accept the concept of prevention of thalassaemia if it were seen to be compatible with religious as well as cultural beliefs. Therefore it is important to consider the views of religious scholars, doctors, and the affected families. Two renowned religious scholars in Pakistan, when asked to give an opinion on the subject of permissibility of prenatal diagnosis for thalassaemia gave a clear verdict that it is permissible provided termination of pregnancy is carried out before 120 days (17 weeks) of pregnancy. Whether the time period specified is gestation or the embryonic age is not clear. But as a general policy we have taken the upper limit as the fetal age determined by ultrasound examination. Both the scholars had based their opinion on the above quoted Quranic verse and the Hadith.

Obstetricians have different views on termination of pregnancy. The two obstetricians who did CVSs in this study are of the opinion that termination can be done at any stage of pregnancy regardless of religious interpretations. They have in fact done CVS for couples reporting late and have also terminated pregnancies in such cases. Although there is no legislation in Pakistan defining an upper limit of permissibility of termination, it is important to work within the framework defined by consensus of opinion. We have not encountered criticism from any religious or social organization since introducing the service for prenatal diagnosis. However if a debate on this subject should arise in future,

the service can be defended if it has the backing of prominent religious scholars. Leaving such sensitive issues to each individual's own judgement in the absence of consultation with religious leaders and the community can have harmful effects for the whole programme.

Several couples in this study who were hesitant in using prenatal diagnosis were greatly relieved to learn that Islam permits termination of pregnancy under special circumstances. During the two study years three couples requested prenatal diagnosis after the 17th week of gestation. They were counselled and informed about the Islamic view on terminating a pregnancy at this stage. All three couples were greatly disappointed to know about this, but all opted to refrain from using the test.

Most of the couples who requested prenatal diagnosis in this study had little hesitation in terminating a pregnancy. Only 3/42 (7%) couples refused termination on religious grounds. An important reason for the high rate of acceptance of termination is that the couples had already made up their minds before requesting the test. It is likely that those who had reservations about the acceptance of termination had avoided the test. Most of the prospectively interviewed couples (87%) would have a termination of pregnancy if required in future.

Cost of prenatal prenatal diagnosis:

There is no allocation for thalassaemia in the National health budget and the affected families have to bear the cost themselves. Therefore the cost of treatment and diagnostic procedures is of fundamental importance in Pakistan. It was observed that the socio-economic status of over 2/3rd of the couples whose children were receiving treatment at a centre in Lahore was low (income <Rs. 5000 per month). This is also reflected by the response of 24% of the couples who would be willing to use prenatal diagnosis only if it were done free of cost. The total cost of one prenatal diagnosis including CVS, laboratory diagnosis and possible termination in selected cases is Rs. 3600 ($90) by the standard method and it can be reduced to Rs. 3000 ($75) by the multiplex ARMS method. The cost is very low as compared to $ 900-1000 per diagnosis in the UK (Petrou et al, 1990). This is mainly because of the very cheap cost of labour in Pakistan. Our experience shows

that even at such a low cost many patients are unable to afford the test. Clearly there is a need to subsidize the cost of prenatal diagnosis by the government or NGOs.

National thalassaemia prevention programme:

Short-term measures:

The "ideal" approach for thalassaemia prevention in Pakistan is a long way in the future. The most important first step would be the organization and utilization of the existing resources. The NGOs have a very important role to play. From the point of view of prevention, the most effective simple and cheap step would be to focus on informing the affected families at the treatment centres about carrier screening and prenatal diagnosis. This step alone may bring about 50% reduction of new births in the families who come to treatment centres (Alwan and Modell 1997). Offering prenatal diagnosis to the couples would further reduce the birth rate. However, the cost of prenatal diagnosis might have to be subsidized to make its full utilization.

Long-term measures:

The long term solution for thalassaemia lies in the adoption of a National policy. A suggested plan for the programme is presented in Fig: 10.5. This includes establishment of a cell for prevention and control of genetic disorders in the ministry of health and the appointment of a National programme co-ordinator who should co-ordinate the activities of the Governmental and the non-governmental organizations. A joint approach for all genetic disorders may be more appropriate to get Government support. Separate expert panels should be appointed under the supervision of the National co-ordinator to organize prevention and treatment activities of the important genetic disorders including thalassaemia.

The expert panel on treatment for thalassaemia should include paediatricians, haematologists, and psychologists. The panel should formulate and ensure adoption of a uniform protocol for thalassaemia management and do research studies on new and cheaper treatment options. For example the trial of oral iron chelators can be a useful study. Provision of screened blood for transfusion should be ensured by compulsory

screening of blood for hepatitis and HIV. A regular audit of the treatment facilities at the centres run by NGOs and charity organizations should be mandated.

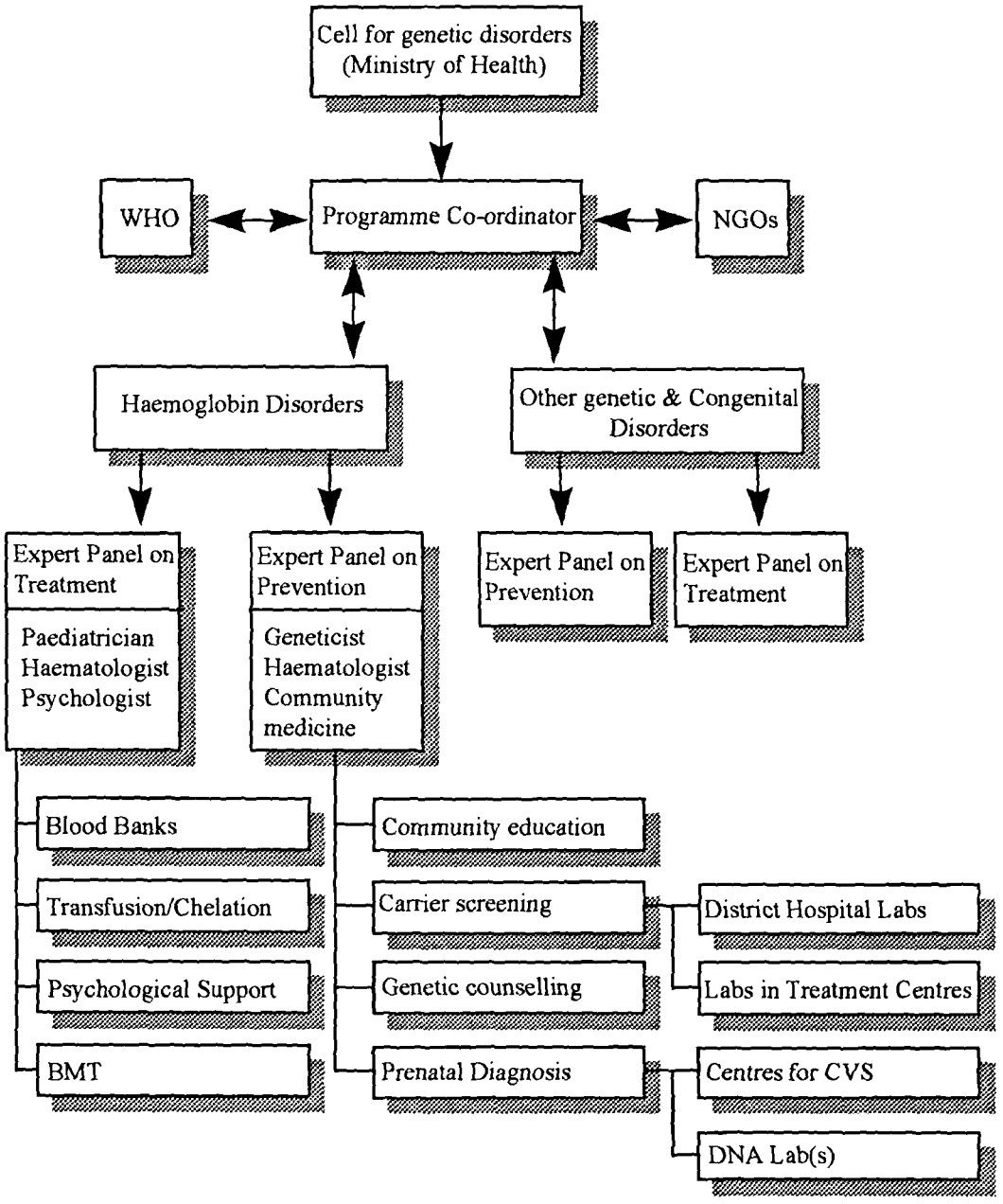


Fig: 10.5. A suggested layout of a National plan for prevention and control of genetic disorders in Pakistan.

The panel on prevention should comprise geneticists, haematologists, and experts on public health and community medicine. The National plan for prevention should include training of manpower and establishment of diagnostic centres. Basic facilities for screening of haemoglobin disorders should be established at each district hospital laboratory. Additional units for carrying out screening of haemoglobin disorders should also be established at each treatment centre. The latter should be equipped with sufficient staff to carry out screening in the families and provide genetic counselling to the affected couples and the carriers.

A centre for carrying out CVS should be established at each thalassaemia treatment centre. The samples collected at these centres can be transported to a central DNA laboratory. A DNA laboratory should also be established in the South of the country. The two laboratories should be able to handle the prenatal diagnostic work for the whole country.

Monitoring the outcome of programme:

The success of the programme can be monitored at regular intervals by evaluating the following parameters (Alwan and Modell 1997) at the treatment centres:

1. Improvement in survival of patients monitored through a central registry of the number and age distribution of patients.
2. Improvement in quality of life of the patients (e.g. growth, school attendance etc.).
3. Reduction in birth prevalence of new cases (also assessed through patient register).
4. Documenting the circumstances of the birth of new thalassaemic children.
5. Impact on the family life of the parents (assessed by measuring the number of healthy children they have relative to the population norm).

Funding for the thalassaemia prevention programme:

In view of the meagre health budget it is unlikely that a major share for funding would be provided by the Government. Therefore fund raising by voluntary donations and NGOs would be important. A National Thalassaemia Fund can be established. The help of

philanthropists and the Zakat Foundation, which is already sponsoring many useful projects in the health sector, can significantly contribute towards initiating a programme as well as its recurring expenditures. Zakat is a Muslim tax of 2.5% that is levied on the total income and fixed assets of the well to do Muslims. The beneficiaries of the Zakat fund are the poor and deserving people.

Cost and benefit of various policies for thalassaemia:

For the purpose of comparing cost effectiveness three policies for thalassaemia in Pakistan are considered. Policy-1 is based on providing only treatment facilities but no measures for prevention. Policy-2, in addition to treatment, includes retrospective genetic counselling and provision of prenatal diagnosis for all future pregnancies. Policy-3, in addition to the features of Policy-2, includes screening the index families with a view to prospectively identify at risk couples and offer prenatal diagnosis. A comparison of the cost of the three policies (Fig: 10.6) clearly demonstrate the benefits of adopting policies that include prevention.

Most of the savings in adopting preventive policies are done on the cost of treatment. Since the Government is not spending anything directly on the treatment of thalassaemics, the cost of initiating a prevention programme is likely to be an additional liability rather than a relief for the Government. Keeping in view the limited health budget and the health priorities of the Government, the role of NGOs and the private sector becomes important. A co-ordinated activity and recruitment of more NGOs working for thalassaemia would be of fundamental importance.

Application of the results to other EMR countries:

Consanguineous marriage and clinically significant haemoglobin disorders are common in most countries of the Eastern Mediterranean Region (Alwan and Modell 1997). Table 10.2 summarizes the frequency of consanguineous marriages and the annual births of children affected with haemoglobin disorders in the EMR countries. The majority population of these countries is Muslim. Prevention programmes for thalassaemia do not exist in any of these countries except Iran. Therefore the response of the population to

various options of prevention is mostly unknown. The cultural social and religious background of the entire EMR has several things in common. However, the socio-economic conditions, education and organization of the health care system varies considerably in different countries of the region.

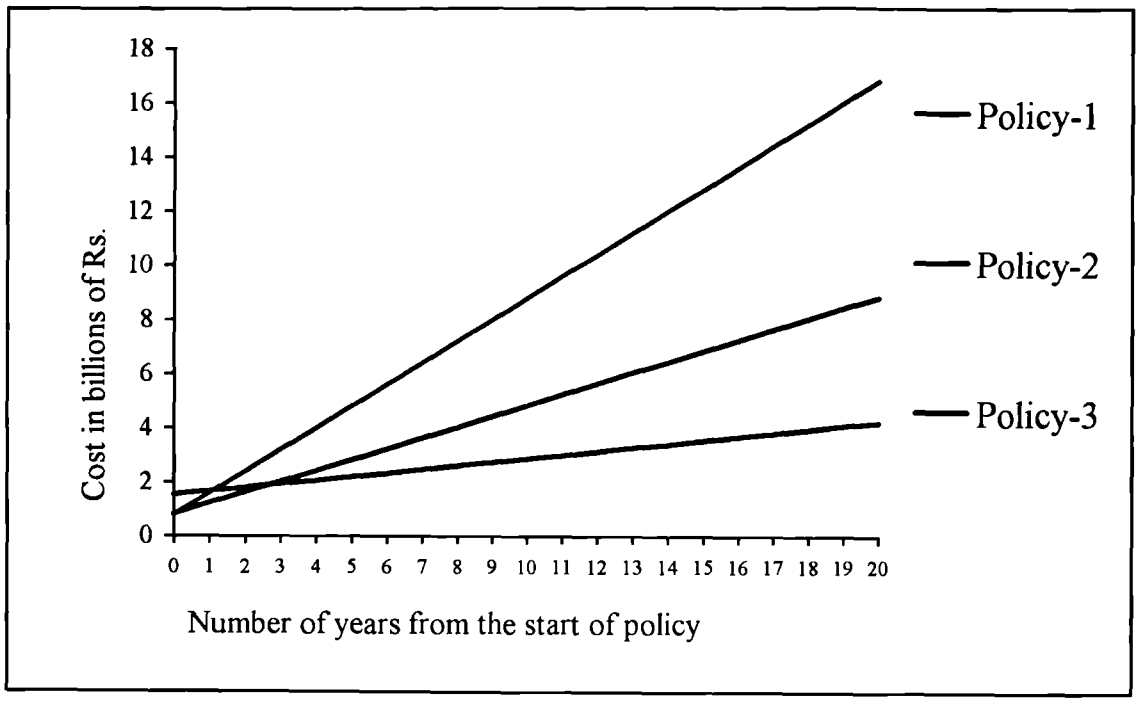


Fig: 10.6. Cost effectiveness of the three policies for thalassaemia in Pakistan. A basic feature of all policies is the provision of adequate treatment facilities to all patients. Policy-1 includes only treatment but no measures for prevention. Policy-2, in addition to treatment, includes retrospective genetic counselling and provision of prenatal diagnosis for all future pregnancies. Policy-3, in addition to the features of Policy-2, includes screening the index families with a view to prospectively identify at risk couples and offer prenatal diagnosis.

The following general guidelines based on the results of this pilot study can be helpful in formulating policies for other EMR countries:

1. Screening the index families is the most cost effective approach to identify at risk couples and unmarried carriers i.e. "premarital testing" well in advance.
2. Screening the index families provides the best opportunity for counselling of the carriers as most members of the family have at least some knowledge of

the illness of the affected child.

3. Antenatal screening may be feasible if most at risk pregnancies can be monitored in the 1st trimester.
4. Prenatal diagnosis is technically feasible by cost effective methods and is also accepted by the affected families provided at risk pregnancies are identified in the 1st trimester.
5. Most religious scholars consider that termination of pregnancy is permissible for a serious genetic disorder provided it is done before 17 weeks of gestation.

Efforts to discourage consanguineous marriages on medical grounds can do more harm than good. The best approach would be to encourage carrier screening in the families at risk and take the information and availability of prenatal diagnosis into account when planning marriages.

Table: 10.2. Frequency of consanguineous marriage and annual number of children affected by haemoglobin disorders in the EMR countries (Based on Alwan and Modell 1997).

Country:	**Total consanguineous marriages:**	**Annual number of children affected with Haemoglobin disorders:**
Bahrain	39%	126
Egypt	29%	808
Iran	37%	1,896
Iraq	58%	1,501
Jordan	50%	97
Kuwait	54%	47
Lebanon (muslims)	30%	110
Libya	?	166
Moroco	?	1,824
Pakistan*	45%	5100
Saudi Arabia	54%	2,845
Sudan	?	2,314
Syria	?	1,043
UAE	54%	46
Yemen	?	1,571

? 20-50% consanguineous marriages
* this study

Appendix-B

QUESTIONNAIRE FOR THE PARENTS OF THALASSAEMICS

Reference:_____

Father's education: [None] [Primary] [Matric] [Above matric]

Mother's education: [None] [Primary] [Matric] [Above matric]

Monthly income of the family: [< Rs. 5,000] [Rs. 5,000-10,000] [>Rs. 10,000]

Total No of children: [_____]

No of thalassaemic children: [_____]

Does thalassaemia affect your family life ? [A little] [Often] [A lot]

Does thalassaemia affect your financial situation ? [A little] [Often] [A lot]

Do you know that prenatal diagnosis is available in Pakistan ? [Yes] [No]

Who told you about prenatal diagnosis ?

[_____]

Did you have a pregnancy in the last 2 years ? [Yes] [No]

If yes, did you request prenatal diagnosis ? [Yes] [No]

If no, then why not ?

[_____]

Would you request prenatal diagnosis in your future pregnancies ?

[_____]

Do you agree with termination of pregnancy for thalassaemia ? [Yes] [No]

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Molecular genetics of β-thalassaemia in Pakistan: a basis for prenatal diagnosis

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Summary. Thalassaemia is the most common inherited disorder in Pakistan and there are very inadequate treatment facilities for over 4000 homozygotes born each year. Prevention of these disorders therefore forms an essential part of the management of this enormous health problem. We have characterized 1216 β-thalassaemia alleles from the five major ethnic groups of Pakistan. The complete spectrum comprised 19 different mutations. There are important ethnic and regional differences in the prevalence of mutations. The five most common mutations, IVSI-5 (G-C) (37·3%), Fr 8–9 (+G) (25·9%), del 619 (7·0%), Fr 41–42 (−TTCT) (6·7%) and IVSI-1 (G-T) (5·4%), constitute 82·3% of the total. Fr 8–9 (+G) is the most common mutation in Northern Pakistan (41·3%), whereas IVSI-5 (G-C) is the most frequent mutation in Southern Pakistan (52·2%). Six subjects with transfusion-dependent thalassaemia major showed only a single mutant allele. One subject with transfusion-dependent thalassaemia major showed a novel 17 bp deletion involving Cd126–131. Our findings provide a comprehensive basis for carrying out prenatal diagnosis of thalassaemia in a geographical area where it is found in high frequency.

Keywords: thalassaemia, mutations, ARMS, DGGE, prenatal diagnosis.

Pakistan has a population of 120 million people. The annual rate of population growth is 3% and almost 40% of the population is below 15 years of age. There are four major ethnic groups: Punjabis and Pathans living in the North, and Sindhis and Baluchis living in the South. In addition, Mohajirs, a large number of immigrants from various parts of Northern India who migrated after the partition of the subcontinent, are settled mainly in the South. Each ethnic group is subdivided into Casts or 'Biradris' of people ranging from a few thousand to millions. There is a very strong tendency for people to marry within their ethnic group, and particularly their 'Biradri'. Another very common custom is marriage between close relatives, especially first cousins (Shami & Zahida, 1981; Darr & Modell, 1988). This peculiar situation results in an unusually high frequency of autosomal recessive disorders.

Infant mortality is falling as the major communicable diseases are coming under control and, consequently, inherited disorders are becoming a recognized problem. β-Thalassaemia is the most common inherited disorder in Pakistan and studies on its carrier frequency have shown an average rate of 5% (Khattak & Saleem, 1992). It is estimated that over 4000 thalassaemic children are born in Pakistan each year, and the available health facilities are inadequate to cope with the number of sick children. The essential part of the approach to this problem is therefore a community-based preventive programme including carrier screening, genetic counselling, and the offer of prenatal diagnosis (Cao, 1987). Prenatal diagnosis will depend on knowledge of the local mutations causing thalassaemia (Kazazian *et al.* 1990), but to date there has been no systematic study of the molecular genetics of thalassaemia in relation to ethnic groups in Pakistan. The only information available is derived from a limited number of Pakistani subjects residing in the U.K. (Varawalla *et al.* 1991a, b). In this study we have investigated a large number of mutant alleles causing β-thalassaemia in the five major ethnic groups of Pakistan.

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MATERIALS AND METHODS

Subjects

The material of this study is derived from a total of 703 unrelated subjects including 184 β-thalassaemia minor cases and 519 cases of transfusion-dependent thalassaemia major. The thalassaemia major patients ranged from 3

months to adult. Representation by ethnic group was as follows: Punjabi 256, Pathan 135, Sindhi 132, Baluchi 89, and immigrants from various parts of India (Mohajirs) 91. The subjects were identified by the Department of Haematology, Armed Forces Institute of Pathology, Rawalpindi, by the Fatimid Foundation's Thalassaemia Treatment Centres at Peshawar, Lahore, Karachi, and by the Hussaini Blood Bank, Karachi.

Mutation analysis

Amplification refractory mutation system (ARMS). The samples were first tested by the method of Amplification Refractory Mutation System (Newton *et al*, 1989; Kwok *et al*, 1990) for the 15 known mutations previously reported in Pakistani and Indian subjects (Varawalla *et al*, 1991a, b). The list and the sequence of the primers used is given in Table I. Subjects with thalassaemia major found to be positive for any mutation were then tested for the presence of the normal allele. Those found to have the normal allele (double heterozygotes) were further tested for other mutations. All of the homozygotes and double heterozygotes were counted as representing two thalassaemic alleles.

PCR was carried out on a 25 μl reaction mixture containing 5 pmol of each primer, 0·3 units of Taq polymerase (Advanced Biotechnologies, U.K.), 30 μM of each dNTP (Boehringer Manheim), 10 mmol Tris HCl (pH 8·3), 50 mmol KCl, 1·5 mmol $MgCl_2$, 100 μg/ml gelatin and 0·3–0·5 μg of genomic DNA. The thermal cycling consisted of 25 cycles of denaturation at 94 C for 1 min, primer annealing at 65 C for 1 min and DNA extension reaction at 72 C for 1·5 min. In the final cycle, the extension reaction was prolonged to 3 min. The PCR products were electrophoresed in 4% NuSieve Agarose gel (Flowgen, U.K.), stained in ethidium bromide and photographed under 302 nm UV light.

Denaturing gradient gel electrophoresis. The samples that did not reveal any mutation by ARMS were subjected to Denaturing Gradient Gel Electrophoresis (DGGE) (Myers *et al*, 1987). Four overlapping regions of the β-globin gene were amplified as described by Cai & Kan (1990). PCR was carried out on a 50 μl reaction mixture containing 10 pmol of each primer, 1·0 unit of Taq polymerase (Perkin Elmer, U.K.), 200 μM of each dNTP (Boehringer Manheim), 10 mmol Tris HCl (pH 8·3), 50 mmol KCl, 1·5 mmol $MgCl_2$, 100 μg/ml gelatin and 0·3–0·5 μg of genomic DNA. The thermal cycling regimen consisted of initial denaturation for 5 min at 95 C and then 40 cycles of denaturation at 94°C for 30 s, primer annealing at 55 C for 30 s, and DNA extension

Table I. List of the mutations screened and the ARMS primers used for their identification.

Mutation	Primer	Used with	Fragment size
IVSI-5 (G-C)	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG	3	285
IVSI-5*	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC	3	285
Fr 8–9 (+G)	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC	3	215
Fr 8–9*	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACT	3	214
IVSI-1 (G-T)	TTA AAC CTG TCT TGT AAC CTT GAT ACG AAA	3	281
IVSI-1*	GAT GAA GTT GGT GGT GAG GCC CTG GGT AGG	4	450
Fr 41–42 (–TTCT)	GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT	3	439
Fr 41–42*	GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA	3	443
Del 619 bp	CAA TGT ATC ATG CCT CTT TGC ACC	2	242
Cd 15 (G-A)	TGA GGA GAA GTC TGC CGT TAC TGC CCA GTA	4	500
Cd 15*	TGA GGA GAA GTC TGC CGT TAC TGC CCA GTG	4	500
Cd 5 (–CT)	ACA GGG CAG TAA CGG CAG ACT TCT CCG CGA	3	205
Cd 5*	ACA GGG CAG TAA CGG CAG ACT TCT CCG CAG	3	207
Cd 30 (G-C)	TAA ACC TGT CTT GTA ACC TTG ATA CCT ACG	3	280
Cd 30 (G-A)	TAA ACC TGT CTT GTA ACC TTG ATA CCT ACT	3	280
Cd 30*	TAA ACC TGT CTT GTA ACC TTG ATA CCT ACC	3	280
Fr 16 (–C)	TCA CCA CCA ACT TCA TCC ACG TTC ACG TTC	3	238
IVSII-1 (G-A)	AAG AAA ACA TCA AGG GTC CCA TAG ACT GAT	3	239
IVSII-1*	AAG AAA ACA TCA AGG GTC CCA TAG ACT GAC	3	634
Cd 26 (G-T)(Hb-E)	TAA CCT TGA TAC CAA CCT GCC CAG GGC GTT	3	278
Cap +1 (A-C)	ATA AGT CAG GGC AGA GCC ATC TAT TGG TTC	4	567
Fr 47–48 (+ATCT)	ATA ACA GCA TCA GGA GTG GAC AGA TAG ATC	3	467
1VS1–25 (25b del)	CTC TGG GTC CAA GGG TAG ACC ACC AGC ATA	3	354
1. Control	CAA TGT ATC ATG CCT CTT TGC ACC		
2. Control	GAG TCA AGG CTG AGA GAT GCA GGA		
3. Common	ACC TCA CCC TGT GGA GCC AC		
4. Common	CCC CTT CCT ATG ACA TGA ACT TAA		

*Primers for the normal alleles.

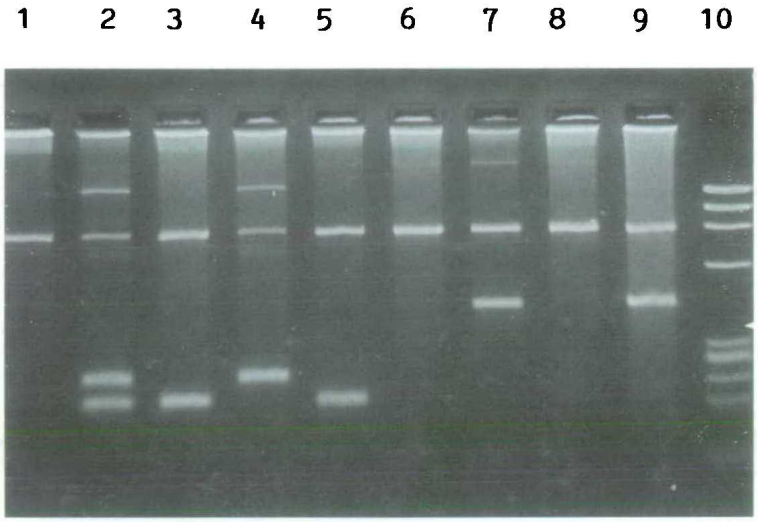

Fig 1. Ethidium bromide stained agarose gel electrophoresis of ARMS PCR products. All lanes show 861 bp amplified products of PCR internal control. Lanes 2, 3 and 5 show 215 bp fragment of Fr 8–9 mutation. The sample in lane 2 also has a 242 bp fragment representing del 619. Lanes 7 and 9 show 439 bp fragment of Fr 41–42 mutation. Lane 10 contains Hae III digest of ØX174.

reaction at 70 C for 1 min. In the final cycle, the extension reaction was prolonged to 3 min. At the end of thermal cycling 10 μl of the amplified product was run on 2% agarose gel to determine the quality of amplification.

DGGE was carried out on 7% polyacrylamide gel containing 32% formamide and 5·6 M urea as denaturants. Varying degrees of gradients ranging from 25% to 80% were prepared by a gradient mixer. The gels were run at 60 C overnight at 40 V.

Genomic sequencing. The region of the β-globin gene shown by DGGE to have a mutation was amplified by a set of primers flanking the mutation. 5 μl of the amplified product was incubated at 37°C for 15 min with 10 units of Exonuclease-I and 2 units of Shrimp Alkaline Phosphatase (United States Biochemical). The Exonuclease-I removes residual single-stranded primers and any extraneous single-stranded DNA produced during PCR. The Shrimp Alkaline Phosphatase removes the remaining dNTPs from the PCR mixture which would interfere with labelling reactions of sequencing. The enzymes were inactivated by heating at 80 C for 15 min.

DNA sequencing was done by the dideoxy chain termination method (Sanger *et al*, 1977). The Sequenase Version 2.0 DNA sequencing kit (United States Biochemical) was used. The enzyme-treated double-stranded amplified product was denatured at 100 C for 2 min with 10 pmol of an internal sequencing primer. The mixture was cooled by placing on ice for 5 min. Labelling was done using 5 μCi ^{35}S-dATP (Amersham, U.K.) and 2 units of Sequenase Version 2.0. The labelled DNA was run on 6% polyacrylamide gel containing 7 M urea at a constant power of 50 W for 2–3 h. The results were read after autoradiography for 48 h.

RESULTS

A total of 1222 mutant alleles were investigated. It was possible to characterize a β-thalassaemia mutation in 1216 alleles. The results of ARMS, DGGE, and genomic sequencing are shown in Figs 1, 2 and 3, respectively. Six transfusion-dependent thalassaemia major cases revealed only one mutation. It was not possible to demonstrate the second mutation even after sequencing the entire β-globin gene and its immediate 5′ and 3′ flanking regions. The 1216 mutant alleles comprised of 19 different mutations. The results of mutations in different ethnic groups is presented in Table II. The five most common mutations, i.e. IVSI-5 (G-C), Fr 8–9 (+G), IVSI-1 (G-T), del 619 bp, and Fr 41–42 (–TTCT) accounted for 82·3% of the alleles. Many regional and ethnic differences in the distribution of mutations were noted (Fig 4). Fr 8–9 (+G) was found to be the most common mutation in Northern Pakistan. The two major ethnic groups of this region, i.e. Pathans and Punjabis, had Fr 8–9 (+G) in 49·1% and 37·2% of the alleles respectively. IVS1-5 (G-C) was found to be the predominant mutation in Southern Pakistan. Sindhis, the major ethnic group of Southern Pakistan, had IVSI-5 (G-C) in 43·9% and Baluchis had IVS1-5 (G-C) in 76·2% of the alleles. Del 619 bp was

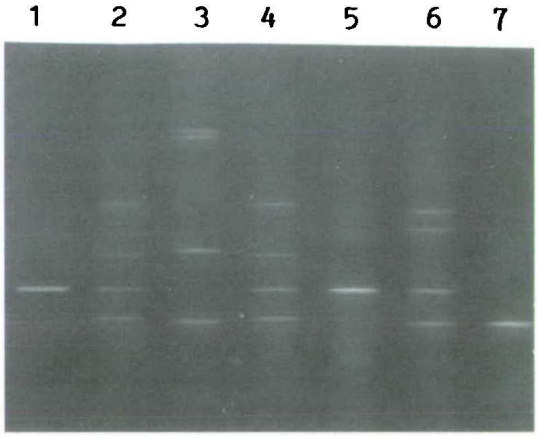

Fig 2. Ethidium bromide stained DGGE of a 286 bp fragment of β-globin gene extending from position –123 to Cd24. Samples in lanes 1 and 5 are homozygous for the normal sequence. Lanes 2 and 4 are heterozygous for a polymorphism at Cd2. Lane 3 is heterozygous for Cap+1 mutation. Lane 6 is heterozygous for Cd15 and lane 7 is homozygous for polymorphism at Cd2.

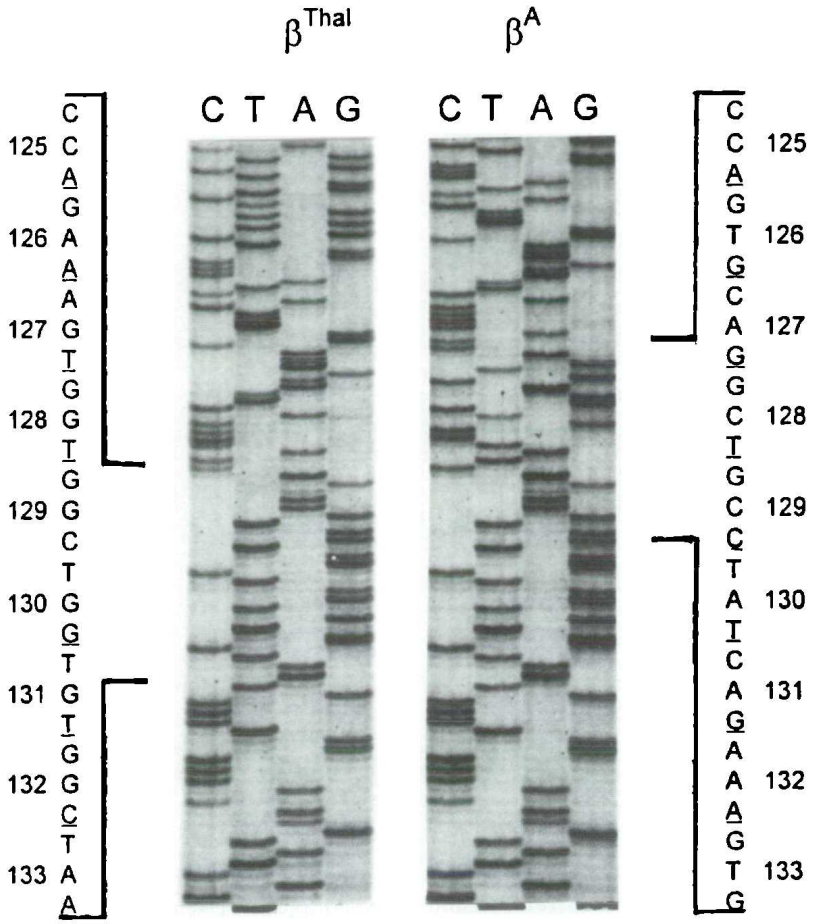

Fig 3. DNA sequencing gel showing the non-coding strand of exon III containing the 17 bp deletion. Sequence of the normal DNA is also shown.

Table II. Prevalence of β-thalassaemia mutations in the major ethnic groups of Pakistan.

Mutation	Punjabi	Pathan	Sindhi	Baluchi	Mohajir	All
Common mutations						
IVSI-5 (G-C)	107 (27·2%)	27 (12·9%)	114 (43·9%)	131 (76·2%)	75 (41·4%)	454 (37·3%)
Fr 8–9 (+G)	146 (37·2%)	103 (49·1%)	29 (11·2%)	14 (8·1%)	23 (12·7%)	315 (25·9%)
Del 619 bp	14 (3·6%)	4 (1·9%)	36 (13·9%)	2 (1·2%)	29 (16·0%)	85 (7·0%)
Fr 41–42 (–TTCT)	36 (9·2%)	18 (8·6%)	16 (6·2%)	1 (0·6%)	11 (6·1%)	82 (6·7%)
IVSI-1 (G-T)	19 (4·8%)	4 (1·9%)	33 (12·7%)	2 (1·2%)	7 (3·9%)	65 (5.4%)
Uncommon mutations						
Cd 15 (G-A)	14 (3·6%)	13 (6·2%)	5 (1·9%)	9 (5·2%)	8 (4·4%)	49 (4·0%)
Cd 30 (G-C)	15 (3·8%)	1 (0·5%)	19 (7·3%)	3 (1·7%)	4 (2·2%)	42 (3·5%)
Cd 5 (–CT)	11 (2·8%)	16 (7·6%)	0 (0·0%)	1 (0·6%)	2 (1·1%)	30 (2·5%)
Fr 16 (–C)	6 (1·5%)	8 (3·8%)	6 (2·3%)	6 (3·5%)	3 (1·7%)	29 (2·4%)
Cap+1 (A-C)	9 (2·3%)	8 (3·8%)	0 (0·0%)	0 (0·0%)	3 (1·7%)	20 (1·6%)
Hb-E	3 (0·8%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	10 (5·5%)	13 (1·1%)
Cd 30 (G-A)	3 (0·8%)	2 (1·0%)	0 (0·0%)	2 (1·2%)	4 (2·2%)	11 (0·9%)
IVSII-1 (G-A)	6 (1·5%)	1 (0·5%)	0 (0·0%)	1 (0·6%)	2 (1·1%)	10 (0·8%)
Rare mutations						
–88 (C-T)	1 (0·3%)	2 (1·0%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	3 (0·3%)
IVSI-1 (G-A)	1 (0·3%)	0 (0·0%)	1 (0·4%)	0 (0·0%)	0 (0·0%)	2 (0·2%)
Fr 47–48 (+ATCT)	2 (0·5%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	2 (0·2%)
Fr 126–131 (–17 bp)	0 (0·0%)	2 (1·0%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	2 (0·2%)
Cd 39 (C-T)	0 (0·0%)	1 (0·5%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	1 (0·1%)
IVSI minus 25	0 (0·0%)	0 (0·0%)	1 (0·4%)	0 (0·0%)	0 (0·0%)	1 (0·1%)
Total	393 (100%)	210 (100%)	260 (100%)	172 (100%)	181 (100%)	1216 (100%)

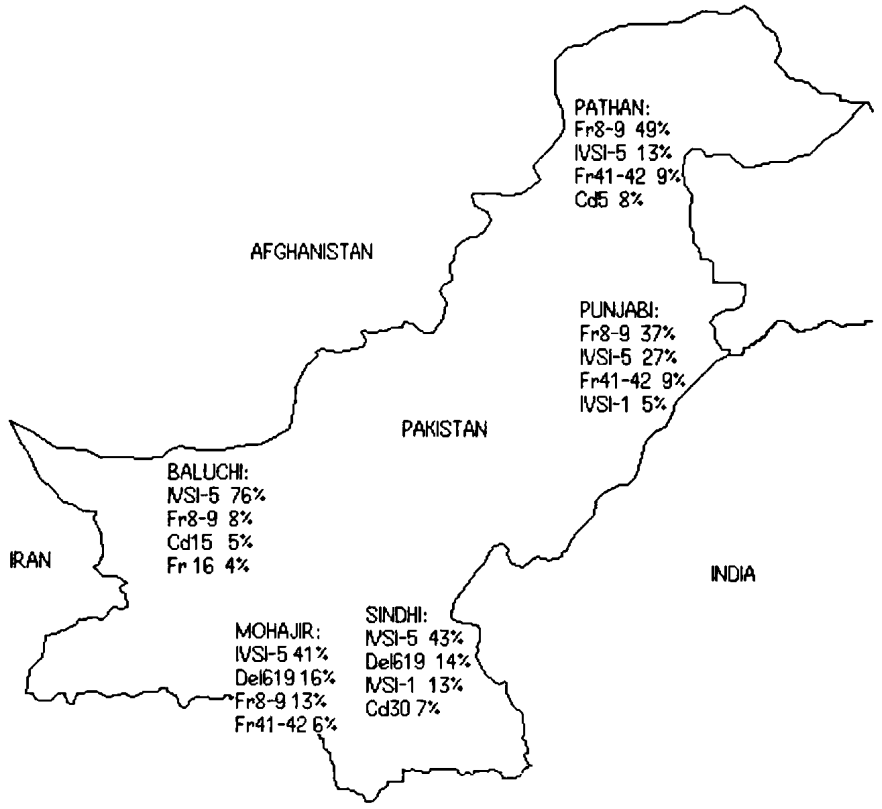

Fig 4. Distribution of β-thalassaemia mutations in different regions and ethnic groups of Pakistan.

seen predominantly in immigrants from India (16·0%) and in native Sindhis (13·9%). Cd5 (−CT), Cd15 (G-A) and Cap+1 (A-C) were seen predominantly in Northern Pakistanis. Fr47/48 (+ATCT), a mutation previously found in a Pakistani subject (Dr Mary Petrou, personal communication), was detected in a Punjabi subject who had thalassaemia intermedia phenotype. Three subjects had typical Mediterranean mutations including IVS1-1 (G-A) and Cd39 (C-T). A Pathan patient, who had consanguineous parents and had severe transfusion-dependent thalassaemia, was found to be homozygous for a novel β-thalassaemia mutation resulting in 17 bp deletion extending from Cd126 to Cd131 (-TGCAGCCTGCCTATCAG) (Fig 3).

The relationship between parental consanguinity and inheritance of mutations in the subjects with thalassaemia major, excluding the six subjects where only one mutant allele was identified, is presented in Table III. Out of the 513 subjects, 315 (61·4%) were offsprings of first cousin marriages. Another 89/513 (17·3%) were born to parents who were related but were not first cousins (relationship not beyond second cousins). In the consanguineous group 330/404 (81·7%) subjects had inherited the same mutation from both parents. The remainder were double heterozygotes for two different mutations. In the non-consanguineous group only 47/109 (43·1%) had inherited the same mutation from both parents.

Table III. Relationship between consanguinity and inheritance pattern of thalassaemia mutations in individuals with two copies of β-thalassaemia genes.

	First cousins			Related but not first cousins			Unrelated		
Ethnic groups (*n*)	Same mutation	Different mutation	Total	Same mutation	Different mutation	Total	Same mutation	Different mutation	Total
Punjabi (137)	81 (83·5%)	16 (16·5%)	97	15 (71·4%)	6 (28·6%)	21	10 (52·6%)	9 (47·40%)	19
Pathan (75)	42 (93·3%)	3 (6·7%)	45	4 (30·8%)	9 (69·2%)	13	7 (41·2%)	10 (58·8%)	17
Sindhi (128)	77 (90·6%)	8 (9·4%)	85	10 (55·6%)	8 (44·4%)	18	7 (28·0%)	18 (72·0%)	25
Baluchi (83)	54 (87·1%)	8 (12·9%)	62	11 (84·6%)	2 (15·4%)	13	7 (87·5%)	1 (12·5%)	8
Mohajir (90)	24 (92·3%)	2 (7·7%)	26	12 (50·0%)	12 (50·0%)	24	16 (40·0%)	24 (60·0%)	40
All groups (513)	278 (88·3%)	37 (11·7%)	315	52 (58·4%)	37 (41·6%)	89	47 (43·1%)	62 (56·9%)	109

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The results of thalassaemia prevention programmes in the Mediterranean (Cao, 1987) have provided a new incentive to overcome the problem of thalassaemia in countries such as Pakistan. Experience in the Mediterranean has shown that preventive programmes for thalassaemia based on heterozygote carrier detection and genetic counselling alone are not effective in reducing the incidence of new births of children with thalassaemia major (Barrai & Vullo, 1980). Prenatal diagnosis of thalassaemia has given a new dimension to thalassaemia prevention. In order to carry out prenatal diagnosis it is most important to have a comprehensive knowledge of the prevalent mutations (Kazazian *et al*, 1990). The molecular genetics of thalassaemia has been studied extensively in subjects from the Indian subcontinent (Kazazian *et al*, 1984; Thein *et al*, 1988; Varawalla *et al*, 1991a, b); however, most of this work has been done on families settled in the West. There has been no well-planned study on the ethnic groups in Pakistan. This study gives a comprehensive picture of the molecular genetics of β-thalassaemia in Pakistan. The spectrum of mutations in all of the ethnic groups studied is heterogenous, and we have found 19 different mutations. An important reason for heterogeneity of the mutations appears to be the geographic location of Pakistan, particularly Northern Pakistan, which has been the gateway for most invasions of the Indian subcontinent. There is a strong possibility that many of the mutations were brought to this region by population migrations.

The results of this study partly conform with a previous study on immigrant Pakistanis who migrated to U.K. from some areas of Northern Pakistan (Varawalla *et al*, 1991a, b); however, we have found a much wider spectrum of mutations. In addition, del 619, previously reported to be present in 56% of Sindhis (Thein *et al*, 1984; Varawalla *et al*, 1991a), represented only 13·9% of alleles from the native Sindhi subjects. Previous studies showing a high prevalence of del 619 were probably carried out on selected groups of patients and were not a true representative of the Sindhi population. We have identified a novel 17 bp deletion in exon III of β-globin gene. The deletion is located between two copies of a CAG sequence present in Cd125/126 and Cd131. It is not possible to ascertain whether the actual breakpoint is before or following the CAG sequence in Cd 125/126. In either case Cd 125 is left intact. Sequence characteristics, such as a reiterated nucleotide sequence of two to eight base pairs (e.g. CAG in this case), separated by a few nucleotides, appear to be involved in almost all small deletions leading to haemoglobin disorders (Bunn & Forget, 1986). As a result of the deletion the reading frame is shifted and a premature stop signal (TAA) is encountered in the new Cd 133. Another interesting aspect of this study is the finding of six subjects who had transfusion-dependent thalassaemia and yet possessed only one thalassaemic allele. It is suspected that transfusion dependency in these apparently heterozygous subjects may in part be due to co-inheritance of triplicated α-globin genes (Garewal *et al*, 1994).

The relationship of mutation pattern and consanguinity shows that mutation analysis in consanguineous couples would be considerably easier, because there is over an 80% chance that both the parents in such couples would have the same mutation. It should be pointed out that the figures of parental consanguinity (61·4%) amongst the thalassaemic families is high because these families are selected and do not represent the overall level of consanguineous marriages in the population. In the Punjabi population, consanguinity has been reported to be around 40% (Shami & Zahida, 1981).

This study provides a comprehensive basis for prenatal diagnosis of thalassaemia in all ethnic groups in Pakistan, which has been introduced recently (Ahmed *et al*, 1994). The service has been offered primarily to parents with affected children. The preliminary results of the first 70 cases diagnosed prenatally at the Armed Forces Institute of Pathology (AFIP), Rawalpindi, indicate that this is technically feasible and is also accepted amongst these families (unpublished observation).

Studies in the U.K. (Petrou, 1994) have shown that British Pakistani couples have particular problems with late termination of pregnancy and only about 23% of couples accept prenatal diagnosis and selective abortion of affected fetuses in the second trimester of pregnancy (Modell *et al*, 1980; Petrou, 1994). However, when counselled in the first trimester of pregnancy, they often make use of prenatal diagnosis. Almost all prenatal diagnoses done at AFIP in Pakistan have been in the first trimester of pregnancy and $>95\%$ of the women with a fetus having thalassaemia major opted for termination of pregnancy. However, there is little awareness amongst the general population and health professionals about the role of prenatal diagnosis in the prevention of thalassaemia. Mediterranean programmes have shown that large-scale community-based thalassaemia prevention programmes can be implemented effectively if they are incorporated into a primary health-care programme (Angastiniotis *et al*, 1986; Cao, 1987). Since no primary health-care system exists in Pakistan, a different approach would be required for this purpose and this needs to be developed.

In a prospective study we will be evaluating the number of couples making use of prenatal diagnosis offered during pregnancy. Studies are also under way to determine suitable approaches for identifying at-risk couples prospectively, with a view to eventually offering them prenatal diagnosis.

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